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The Agricultural Chemical Society of Japan

*Studies on Oxidative Fermentation.** *Part XII*

On the Formation of Rubiginic Acid, a New γ-Pyrone Derivative, and Comenic Acid by Gluconoacetobacter liquefaciens.

By Kō Aida

The Institute of Applied Microbiology, University of Tokyo

Received January 11, 1955

Two substances besides rubiginol, of positive ferric chloride reaction were isolated from a shaking culture of *Gluconoacetobacter liquefaciens*, using glucose as the substrate. One of them was found to be a new γ-pyrone derivative and the composition was concluded to be 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid. The other substance was found to be comenic acid. Assumptions were made with regard to the mechanism of the formation of these γ-pyrone derivatives from glucose.

Introduction

In the previous paper[1),2)], the authors studied the oxidative activities of *Gluconoacetobacter liquefaciens* in surface and shaking cultures. Sugars, such as glucose, fructose, mannose, galactose, xylose, and arabinose were oxidized by this bacteria, and the oxidative products were detected by paper chromatographic method. Separation and identification of oxidative products from glucose were carried out in detail. Aldehyde, formic, acetic, glycolic, tartronic, unknown reducing acid and three kinds of substances which gave a positive ferric chloride reaction were found in the culture liquid of 0.8% yeast extract water containing 9.30% glucose and 2.5% calcium carbonate after 10 days' shaking culture at 30°C. This bacteria also formed 5-ketogluconic acid from glucose by surface culture but it is not sure in the case of shaking culture. One of these substances of positive ferric chloride reaction was isolated as crystals and was found to be a new substance, which was named rubiginol. Its chemical properties were studied and its composition was concluded to be 3,5-dihydroxy-1,4-pyrone[3),4)].

In the present paper, the author isolated the other two substances of positive ferric chloride reaction as crystals and their chemical properties were studied. One of them was found to be a new substance, which was named rubiginic acid, and the other was identified as comenic acid. The composition of rubiginic acid was concluded to be 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid, from which rubiginol was obtained as its decarboxylated product.

Experimental

I. Separation of Substances of Positive Ferric Chloride Reaction.

In order to separate the organic acids formed, the fermentation broth of *Gluconoacetobacter liquefaciens* was subjected to ether extraction after

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* The foregoing title, "Studies on the Oxidative Bacteria" was changed like this.

1) K. Aida, T. Kojima and T. Asai, *J. Agr. Chem. Soc. Japan* **28**, 517 (1954).

2) K. Aida, T. Kojima and T. Asai, *J. Gen. Appl. Microbiol.*, **1**, 18 (1955)

3) K. Aida, *J. Agr. Chem. Soc. Japan*, **28**, 523 (1954).

4) K. Aida, *J. Gen. Appl Microbiol.*, **1**, 30 (1955).

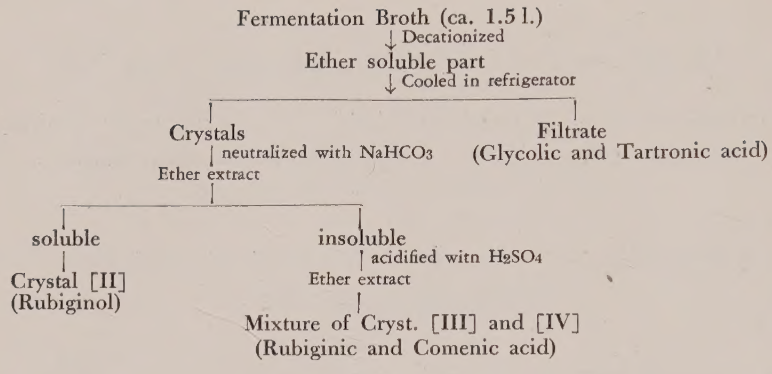
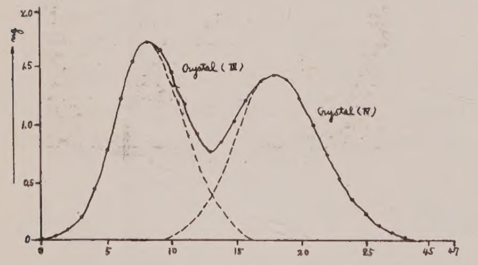


Fig. 1. Separation of Three Substances of Positive Ferric Chloride Reaction

Strain: *Gluconoacetobacter liquefaciens*
Medium: 0 8% yeast extract water containing 9.30% glucose and 2.5% $CaCO_3$.
Incubated by shaking culture for 10 days at 30°.
Separation and identification of othet metabolic products were described in the previous paper[1), 2)]



Tube number (*of 48*)

Fig. 2. The 48 plates counter current distribution of crystal [III] and [IV] which was carried out, using ethylacetate and water as the solvents system.

being decationized with oxalic acid and cation exchange resin (cf. the previous paper[1),2)]). As seen in Fig. 1, three substances of positive ferric chloride reaction were extracted with ether. After 50 hours' extraction, the extracted part was evaporated and while kept in a refrigerator overnight there separated out crystal [II] with a small amount of crystal [III]. Ether extraction was further continued for several days, the above mentioned procedure was repeated again, and there separated out a mixture of crystal [III] and crystal [IV] with a small amount of crystal [II]. Crystal [IV] was most difficult to be extracted, and remained in unextracted part for a long time. As reported in the previous paper[3),4)], rubiginol has phenolic OH groups but no carboxyl group, while other two substances have a carboxyl group. Accordingly, rubiginol was separated from others by ether extraction after dissolving a mixture of them in sodium bicarbonate solution. The other two substances were so similar in their chemical properties that it was not easy to separate them each other. Crystal [III] and [IV] was subjected to 48 plates counter current distribution[5),6)], using ethylacetate and water as the solvents system. Result is shown in Fig. 2. Distribution coefficient (K) of crystal [III] and [IV] calculated were ca. 0.217 and 0.614, respectively. From this result, it was found that the mixture of these two substances would be capable to be separated each other. One litre separate funnels were used for counter current distribution, and the solvent of the plates was evaporated after the purity was checked by paper chromatography. Crystals [III] and [IV] were obtained free from each other by repeating this procedure. The yields of these three substances were low. From 140 g of

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5) S.W. Liebermann, *J. Biol. Chem.*, **173**, 63 (1948).
6) Y. Sumiki, *J. of Antibiotics* (Japan) **2**, 400 (1949).

glucose, 1.2 g of rubiginol, 0.6 g of crystals [III] and 0.5 g of crystals [IV] were obtained.

II. The composition of Rubiginic Acid.

(1) *Properties of Rubiginic Acid.* As the substance corresponding to the crystal [III] was not found in the existing literature, it was named Rubiginic acid. It was obtained as colorless prismatic needles, and decomposes at 230°C (uncorr.) with the evolution of gas. It is soluble in pyridine, dioxane, acetone, methanol, ethanol, water, isopropanol, butanol, glacial acetic acid, ethylacetate, and ether, but scarcely or not soluble in toluol, xylol, benzol, petroleum ether, and ligroin. It is strongly acidic and expels carbon dioxide gas from alkaline carbonate or bicarbonate solution. Its aqueous solution shows an acidic reaction and reduces Fehling's and ammoniacal silver nitrate solution. Light green copper salt precipitates out from aqueous solution by the addition of copper acetate solution. It decolorizes bromine water and potassium permanganate solution. From above mentioned facts, it seemed to be an organic acid, which contains at least one carboxyl group, one carbonyl group and one double bond.

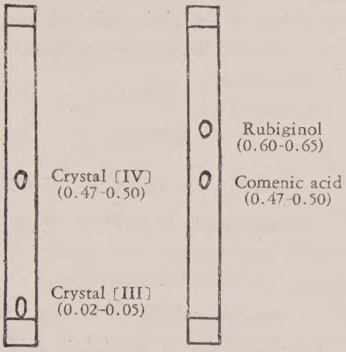
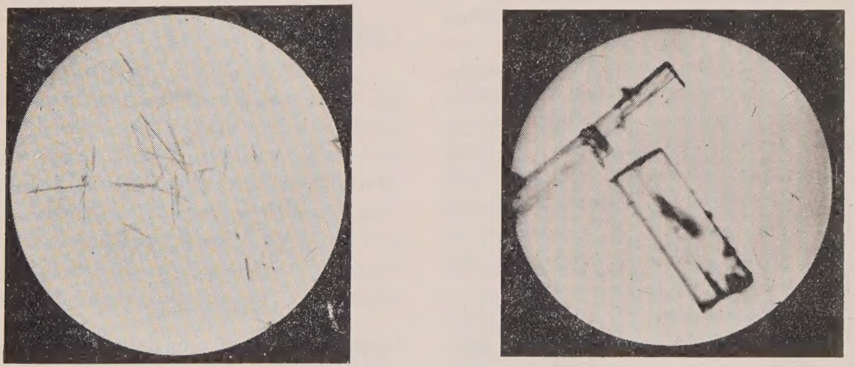


Fig. 3. Paper chromatograms of γ-pyrone derivatives formed by *Gluconoacetobacter liq.*
Samples were chromatographed on Tōyō Filter Paper No. 50 with *n*-butanol 4, formic acid 1.5, water 1, solution and spots were detected with 1% ferric chloride solution.

(2) *Elemental analysis.* After it was dried by



Rubiginic acid ×350 Methylated compound of rubiginic acid ×350

Fig. 4. Photographs of crystals

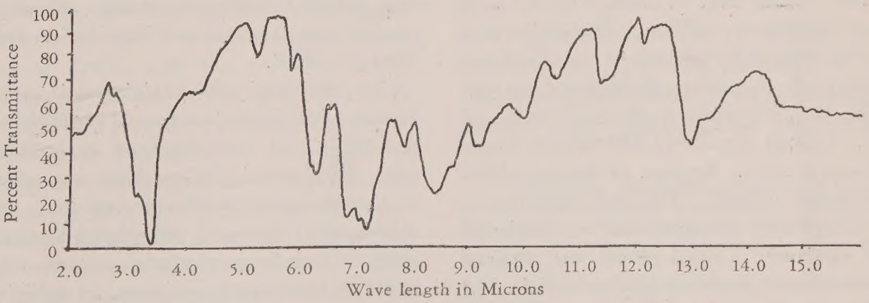


Fig. 5. Infrared absorption spectrum of rubiginic acid

Abderhalden's apparatus in vacuo at 100°C, elemental analysis was conducted. It was free from nitrogen and sulfur.

Analysis. Found: C, 41.93, 42.14; H, 2.58, 2.63; Calcd. for $C_6H_4O_6$: C, 41.86; H, 2.26%

(3) *Determination of Molecular Weight.* Molecular weight was calculated as 175 ($C_6H_4O_6$= 172) from its potentiometric titration curve. Because it was not soluble in camphor, Rast's method was not adopted. Molecular formula of rubiginic acid was then concluded to be $C_6H_4O_6$.

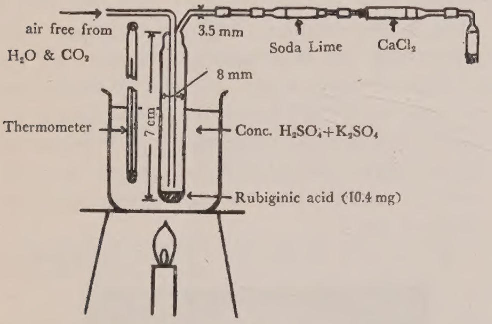
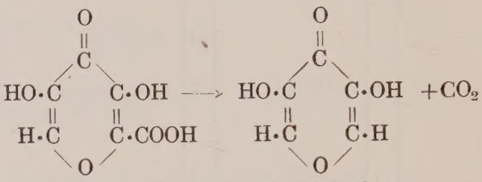


Fig. 6. Heat decomposition of rubiginic acid.

(4) *Infrared Absorption Spectrum of Rubiginic Acid.* Infrared recording spectrophotometer, Baird model B No. 143 was used. Infrared absorption spectrum of rubiginic acid is shown in Fig. 5. The absorption spectrum of infrared ray suggested the existence of a secondary or phenolic OH group, possibly a conjugated carbonyl and carboxyl group in its structure.

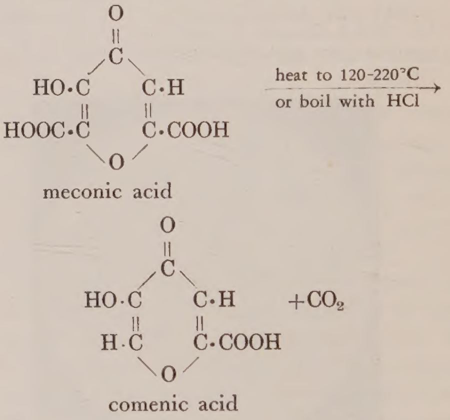
(5) *Heat Decomposition of Rubiginic Acid.* 10.4 mg of rubiginic acid was taken in a little glass tube, which was connected to another glass apparatus as shown in Fig. 6. This was heated to 230–240° in the bath of conc. sulfuric acid dissolving patassium sulfate. Rubiginic acid decomposed with the evolution of gas which was introduced to the apparatus of elemental analysis by passing the air free from carbon dioxide and moisture. Carbon dioxide (2.230 mg) was found to have been evolved. Residue of decomposition products melted clearly. This was dissolved in sodium bicarbonate solution and was subjected to ether extaction. Crystal (4 mg) was obtained from extacted part, and identified as rubiginol by its melting point and paper chromatography. Therefore, it was concluded that one mole of rubiginic acid decomposed to one mole of rubiginol with the liberation of one mole of carbon dioxide. Yield of carbon dioxide caught was calculated as 84% of theoretical. Accordingly, the composition of rubiginic acid was assumed to be 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid as shown in Fig. 7.



Rubiginic acid Rubiginol

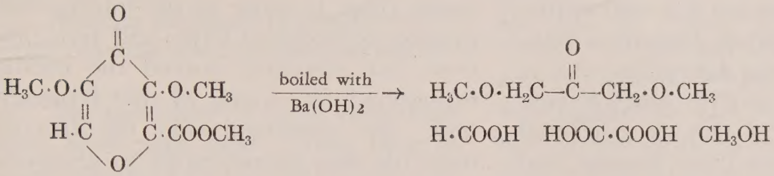
Fig. 7. Decomposition of rubiginic acid

(6) *Decarboxylation of Rubiginic Acid by conc. Hydrochloric Acid.* As the next reaction is well known,7) 20 mg of rubiginic acid was dissolved in 30 ml. of conc. hydrochloric acid and boiled with reflux cooler for 5 hours. After neutralized with sodium bicarbonate solution, decomposition product was extracted and identified as rubiginol, yield ca. 60%.



(7) *Methylation of Rubiginic acid and Decomposition of Methylated Compound of Rubiginic Acid.* Rubiginic acid was dissolved in methanol and ether solution of diazomethane was added until it showed no ferric chloride reaction. After the solvent was evaporated, methylated compound was recrystallized from methanol, which melted at 138°C. Methylated compound of rubiginic acid was dissolved in barytha water and boiled for 30

min. with reflux cooler and decomposition products were analyzed. The next reaction was expected from the assumed composition of rubiginic acid.



The distilled fraction of decomposition products reduced Fehling's and ammoniacal silver nitrate solution. 2,4-Dinitrophenylhydrazine solution, 0.7% in 6 N hydrochloric acid, was added to it, and there precipitated out yellow orange crystals of 2,4-dinitrophenylhydrazone. Recrystallized from methanol, the crystal melted at 121°C and the melting point was not depressed by admixture with 2,4-dinitrophenylhydrazone of dioxyacetone dimethylether. The determination of OCH_3 group was conducted about it.

Analysis. Found: OCH_3 20.68; Calcd. for $C_9H_8O_4N_4$ $(OCH_3)_2$: OCH_3 20.81 %

The residue was acidified with sulfuric acid and extracted with ether, and formic acid was identified in the extracted part. Oxalic acid was not yet identified in it, but Ca-salt, insolube in acetic acid, was obtained and it seemd to be oxalic acid. From the facts mentioned from (1) to (7), it was concluded that the composition of rubiginic acid was 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid.

III. Identification of Comenic Acid.

Crystal [IV] is a strong organic acid and expels carbon dioxide gas from alkaline carbonate or bicarbonate solution. It is a plate crystal and decomposes at 276°C with the evolution of gas. It is hardly soluble in cold water but easily in hot. Soluble in methanol, ethanol, acetone, pyridine, dioxane and ether but not soluble in ligroin, benzol, xylol, toluol, petroleum ether and chloroform. It gives a reddish violet color reaction with ferric chloride solution, and reduces Fehling's and ammoniacal silver nitrate solution weakly. The aqueous solution of the acid decolorizes bromine water and potassium permanganate solution. The substance, dried in vacuo at 100°C gave following analytical data:

Analysis. Found: C, 46.03, 46.11; H, 2.65, 2.73; Calcd. for $C_6H_5O_5$: C, 46.18; H, 2.58%

Sample, 50 mg, was dissolved in methanol and added with ether solution of diazomethane until it showed no ferric chloride reaction. After solvent was evaporated, methylated compound was recrystallized from methanol, which melted at 197°C and did not show a positive ferric chloride reaction and an acidic reaction, yield 95%.

Analysis. Found: C, 52.85; H, 4.25; Calcd. for $C_8H_8O_5$: C, 52.12; H, 4.34; Found: OCH_3, 33.58; Calcd. for $C_6H_2O_3$ $(OCH_3)_2$: OCH_3, 33.69%.

The decomposition point of crystal [IV] does not agree with that of existing literature of comenic acid[7],[8],[9] but melting point of methylated compound of crystal [IV] quite agreed with that of methyl ether of methylcomenate[10]. Then comenic acid was prepared from meconic acid[7], which decomposed at 276°C and the decomposition point was not depressed by admixtrne with crystals [IV]. The *Rf* value of crystal [IV] agreed with that of comenic acid as shown in Fig. 3. Mixed melting point of methylated compound of crystals [IV] and methyl ether of methylcomenate was also not altered, so it was identified as comenic acid.

Discussion

Very little was known about the formation of γ-pyrone derivatives as the metabolic products of microorganisms except kojic acid, which had been discovered by K. Saito[11] in 1907 and whose composition was determined by T. Yabuta[12),13),14)] in 1924. Many species of fungi are known to produce kojic acid from various carbohyrates, but it is noteworthy that with one exception, *Penicillium dalae*, all belong to the genus *Aspergillus*. But in 1933, T. Takahashi and T. Asai[15,16] confirmed that kojic acid was also produced by *Gluconobacter* species from fructose and mannitol but not from glucose. Recently, Kondo[17] reported its formation from sorbose by the same species of oxidative bacteria. A similar γ-pyrone derivative, comenic acid was also found and identified in the fermentation products of galactose by *Gluconobacter liquefaciens*[18),19)].

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[7] F. Beilstein, Handbuch der Organischen Chemie, Dritte umgearbeitete Auflage, Erster Band, Verlag von Leopold Voss, Hamburg und Leipzig, 1893, Seite 779.

[8] T. Takahashi and T. Asai, *J. Agr. Chem. Soc. Japan*, **10**, 604 (1934)

[9] T. Takahashi and T. Asai, *Zentr. Bakt. Abt. II*, **93**, 248 (1936).

[10] T. Yabuta, *J. Agr. Chem. Soc. Japan*, **1**. 1 (1924).

Now the authors, in the course of study on the oxidative metabolic products of *Gluconobacter*, discovered that the three γ-pyrone derivatives, i.e., rubiginic acid, rubiginol, and comenic acid were produced from glucose by *Gluconoacetobacter liq.* and their formation was limited only in the case of shaking culture. The author is now studying the mechanism of their formation. But the facts that in other experiment by the author[20] rubiginic acid, rubiginol, and comenic acid were also produced by *Acetobacter melanogenum* and the strains belonging to it were reported[21] to oxidize glucose via the next pathway, i.e., glucose ⟶ gluconate ⟶ 2-ketogluconate ⟶ 2,5-diketogluconate, suggest the possibility of these compound to be the intermediates of γ-pyrone ring formation by this bacteria. Recently, Ikeda[22] confirmed the formation of glucoson from fructose as the intermediate product of bacterial kojic acid fermentation and moreover proved the enediol formation in the course of that fermentation. He presumed that the fructose molecule may be converted to glucosone at first, then transformed into pyranose form, from which kojic acid may be derived after splitting two molecules of water. The author have yet no conclusive proof to explain the mechanism of rubiginol, rubiginic acid or comenic acid formatino, but Ikeda's hypothesis seems to include a great deal of inforation about the formation of γ-pyrone derivatives from hexoses.

Summary

(1) Besides rubiginol, two substances of positive ferric chloride reaction were isolated from the culture liquid of *Gluconoacetobacter liquefaciens* with a medium of 0.8% yeaet extract water containing 9.3% glucose and 2.5% calcium carbonate.

(2) One of them was found to be a new γ-pyrone derivative and was named rubiginic acid. It was isolated in the shape of prismatic needles and decomposes at 230°C. It gives a reddish violet color reaction with ferric chloride solution and reduces Fehling's and ammoniacal silver nitrate solution weakly, and decolorizes bromine water and potassium permanganate solution. Its molecular formula is $C_6H_4O_6$ and one mole rubiginic acid decomposed to one mole rubiginol with the liberation of one mole

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11) K. Saito, *Botan. Mag. Tokyo*, **21**, 7 (1907).

12) T. Yabuta, *J. Chem. Soc. Tokyo*, **37**, 1185 (1916).

13) T. Yabuta, *J. Chem. Soc. Tokyo*, **37**, 1325 (1916).

14) T. Yabuta, *J. Chem. Soc*, **125**, 575 (1924).

15) T. Takahashi and T. Asai, *J. Agr. Chem. Soc. Japan*, **9**, 55 (1933).

16) T. Takahashi and T. Asai, *ibid.*, **9**, 369 (1933).

17) K. Kondō and S. Wada, *J. Fermentation Technology*, **27**, 331 (1949).

18) T. Takahashi and T. Asai, *J. Agr. Chem. Soc. Japan*, **10**, 604 (1934).

19) T. Takahashi and T. Asai, *Zentr. Bakt. Abt. II*, **93**, 248 (1936).

20) K. Aida, It will be later published.

21) H. Katznelson, S.W. Tannenbaum, and E.L. Tatum, *J. Biol. Chem.*, **204**, 43 (1953).

22) Y. Ikeda, *J. Agr. Chem. Soc. Japan*, **28**, 538 (1954)

carbon dioxide by heating at 230°C or boiling with conc. hydrochloric acid. The composition of rubiginic acid was concluded to be 3,5-dihydroxy-1,4-pyrone-2-carboxylic acid.

(3) Another substance of positive ferric chloride reaction was identified as comenic acid.

(4) The quantities of these substances formed were very little. From 140 g of glucose, about 1.2 g of rubliginol, 0.6 g of rubiginic acid, and 0.5 g of comenic acid were obtained.

Acknowledgements

I wish to express my hearty thanks to Prof. T. Asai for his kind guidance and encouragement in this work and am also indebted to Dr. T. Yabuta, Prof. Y. Sumiki, Prof. K. Sakaguchi, Director of the Institute of Applied Microbiology, University of Tokyo, and Dr. Y. Ikeda for their kind suggestions and Mr. H. Kamata for his help with infrared absorption spectrum, and to microanalysers of the Depertment of Agriculture Chemistry, Faculty of Agriculture, University of Tokyo.

Isolation of Astragalin and Isoquercitrin from Bracken, Pteridium aquilinum

By Toshio NAKABAYASHI

Hiroshima Women's Junior College

Received February 7, 1955

From the alcoholic extract of bracken, *Pteridium aquilinum*, two flavonoid pigments, namely astragalin and isoquercitrin, were isolated in the form of yellowish needles, and further a small quantity of rutin and two unknown flavonoid pigments were detected on the paper chromatogram.

Because some of these pigments have the thiamine-decomposing activity, it is presumed that a portion of the so-called thermostable antithiamine factor of bracken is due to these flavonoid pigments therein.

Since in 1946 Weswig and his co-workers1) observed that bracken fern (*Pteris aquilina*) has the antithiamine activity, many investigators have been studying on this phenomenon.

Matsui2) and Okamoto3) also studied on this phenomenon in 1952 and found that bracken, *Pteridium aquilinum* Kuhn, which was called " Warabi " and was generally used for food in Japan, contained two antithiamine factors. One was according to their theory, a thermolabile factor such as thiaminase which was discovered by Fujita,4) Green,5) and Sealock6) individually, and the other was the non-enzymatic factor which was called "the thermostable antithiamine factor " (henceforth called, for convenience sake, SF) by them.

Recently, Fujita7) and Kawakami8) presumed that the SF of bracken is something like rutin, and in addition they have proved that many polyphenol compounds such as flavonoid pigments, catechol, and tannins have the thiamine-decomposing activity. However the kind and structure of the polyphenol compounds in bracken have not yet been ascertained.

From such a point of view, the author has been engaged in the study to clarify the SF of bracken chemically. By studying the properties of the pigments isolated from bracken, it has been found that at least five flavonoid pigments are present therein. Of these pigments, two have been isolated in the form of yellow and needle crystals. One was recognized as isoquercitrin (quercetin-3-glucoside, m.p. 241~243° $C_{21}H_{20}O_{12} \cdot 1\frac{1}{2}H_2O$) and the other was identified as astragalin (kaempferol-3-glucoside, m.p. 206~207°, $C_{21}H_{20}O_{11} \cdot H_2O$) which had been isolated from the flower of *Astragalus sinicus* L.9) and reported to be present in tea leaves10) by the present author. A small quantity of

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1) P. H. Weswig, Anna May Freed, and J. R. Haag, *J. Biol. Chem.* **165**, 737 (1946).

2) K. Matsui, *Vitamins* 5, 594 (1952).

3) T. Okamoto, *Seikagaku* **24**, 103 (1952) ; *Vitamins* **6**, 26, 30 (1953).

4) A. Fujita and K. Numata, *Seikagaku* **17**, 156 (1943) ; **18**, 63 (1944).

5) R. G. Green, W. E. Carson, and C. A. Evans, *J. Nutr.* **21**, 243 (1941) ; **23**, 165 (1942).

6) R. R. Sealock, A. H. Levermore, and C. A. Evans, *J. Am. Chem. Soc.* **65**, 935 (1943).

7) A. Fujita, *Vitamins* **7**, 1 (1954).

8) T. Kawakami, *Vitamins* **7**, 1069 (1954).

9) T. Nakabayashi, *J. Agr. Chem. Soc. Japan* **26**, 539 (1952).

10) Y. Ōshima and T. Nakabayashi, *ibid.* **27**, 756 (1953).

other three flavonoid pigments were detected on the paper chromatogram, of which one was identical with rutin (quercetin-3-rhamnoglucoside) and the others were unknown.

Moreover it was proved that the flavonoid pigments possessing free orthodihydroxyl group in the side phenyl radical such as quercetin and it glycosides have the marked thiamine-decomposing activity at pH 7.0, 70°. The findings would provide substantial evidence for the possibility that among the flavonoid pigments proved therein, isoquercitrin and rutin may be a portion of the SF of bracken.

Experimental

I. Isolation of Two Pigments: Bracken used in this experiments was collected in Hiroshima in May, 1954. The fresh material (2 kg) without root was hashed and boiled three times with 8 liters portion of 70 per cent methanol for one hour. The filtered brownish methanol extract was concentrated by distillation to 400 cc under reduced pressure. The aqueous extract was cooled and filtered twice in order to remove the insoluble substances. After the filtrate was washed with ether several times, the residual aqueous solution was extracted with ethyl acetate repeatedly until the ethyl acetate layer became colorless. After the yellowish ethyl acetate extract was dried with anhydrous sodium sulfate, ethyl acetate was distilled off under reduced pressure. The residue was dissolved in a small quantity of hot water and the resulting solution covered with toluene was allowed to keep in ice box until the pigment crystallized.

After four months, the yellowish precipitate was collected by filtration, washed with cold water, and then the crude yellowish crystals (0.52 g) were obtained by crystallization from boiling water. The results of the paper chromatography showed that this crude pigment consisted of two flavonoid pigments, therefore the recrystallization from the boiling water was repeated with strict attention. After eighteen recrystallizations, the hot water slightly soluble crystals (0.29 g) and soluble one (0.13 g) were obtained. These two pigments gave respectively one spot on the paper chromatogram (Table I. ii, iii, v.).

II. Identification of Astragalin: The hot water slightly soluble crystals were fine and pale yellow-colored needles (Fig. 1). Their alcoholic solution gave dark brown coloration by adding ferric chloride, gave reddish coloration in both cases when reduced with conc. hydrochloric acid

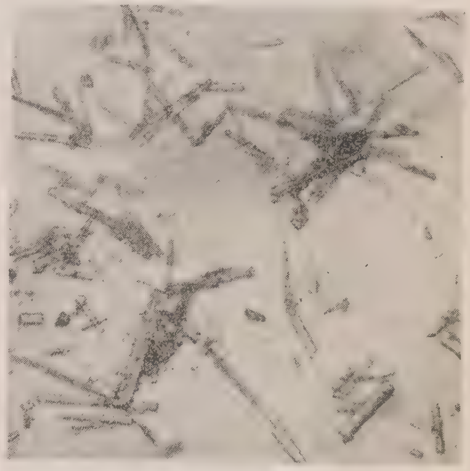

Fig. 1. Astragalin from Bracken (from boiling water) × 120

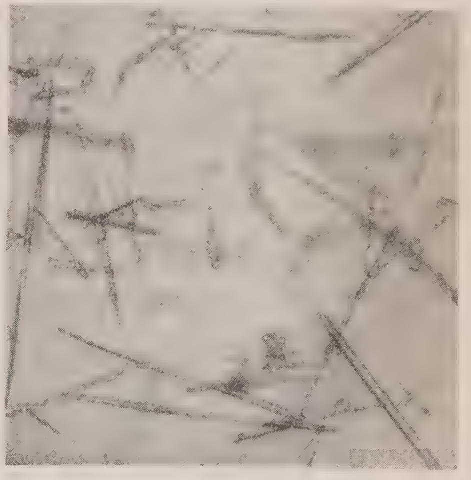

Fig. 2. Heptaacetyl astragalin (from 50% methanol) × 25

and metallic magnesium, and conc. hydrochloric acid and metallic zinc, and yielded yellowish precipitate with lead acetate solution. Its alkali solution presented yellowish coloration. This pigment was readily soluble in ethanol, methanol, and ethyl acetate, soluble in boiling water, and insoluble in ether, acetone, chloroform, and cold water. The results of above stated qualitative tests were entirely similar to astragalin isolated from the flower of *Astragalus sinicus*.

It melted at 206～207° (uncorr.), showed no depression of mixed melting point with recrystallized known astragalin, and gave the same R_F value as astragalin on the paper chromatogram (Table I. iii).

Anal. Found: H, 4.59; C, 53.70; Calcd. for $C_{21}H_{20}O_{11} \cdot H_2O$: H, 4.72; C 54.08%.

On usual acetylation with acetic anhydride and one drop of conc. sulphuric acid, it yielded colorless needles, m.p. 195～197° (uncorr.), when recrystallized from 50 per cent methanol (Fig. 2). No depression of the melting point was observed when this needle was mixed with the recrystallized known heptaacetyl astragalin. The acetate gave no coloration with ferric chloride.

Anal. Found: CH_3CO, 39.91, Calcd. for $C_{21}H_{13}O_{11}(COCH_3)_7$: CH_3CO, 40.85%.

When hydrolyzed with 5 per cent sulphuric acid on a boiling water bath for an hour, 110.0 mg of glycoside yielded 71.0 mg of yellowish needle, which was identified as kaempferol by the paper chromatography (Table I. iv), and 43.7 mg of glucose, which was also identified by the same procedure (Table I. v) and determined by the micro Bertrand method.

According to the following schema:

$$C_{21}H_{20}O_{11} \cdot H_2O \xrightarrow{H_2O} C_{15}H_{10}O_6 \cdot H_2O + C_6H_{12}O_6$$

Recovery	Theoretical :	kaempferol 65.2%, glucose 38.6%
	Found :	kaempferol 64.5%, glucose 39.7%

On recrystallization of aglycone from alcohol, it afforded yellowish needle, m.p. 275～277° (uncorr.). Its acetylation by usual method gave colorless needle, m.p. 185° (uncorr.).

Anal. Found: CH_3CO, 38.01; Calcd. for $C_{15}H_6O_6(COCH_3)_4$: CH_3CO, 37.89%.

Through the mixed melting point determination with known crystals, the recrystallized aglycone and its acetate were severally decided to be kaempferol and tetraacetyl kaempferol. The qualitative tests of aglycone with ferric chloride, conc. hydrochloric acid and magnesium, lead acetate, and alkali were the same with known kaempferol. Moreover the conc. sulphuric acid solution of aglycone gave strong and beautiful green fluorescence under ordinary light, which was the specific reaction of kaempferol.

The absorption spectra of astragalin and kaempferol isolated from bracken were estimated by using Beckman Spectrophotometer and were identical respectively with those of known astragalin and kaempferol (Fig. 4). The maxima of these spectra in ethanol are as follows: Astragalin... 267mμ, 354mμ; Kaempferol... 269mμ, 367mμ.

III. Identification of Isoquercitrin: The hot water soluble crystals were fine and yellow-colored needles (Fig. 3). Its alcoholic solution gave dark green coloration by adding ferric chloride, and developed reddish coloration in both cases when reduced with conc. hydrochloric acid and magnesium, and conc. hydrochloric acid and zinc. The other several qualitative tests and solubilities of the pigment were also entirely similar to those of known isoquercitrin.

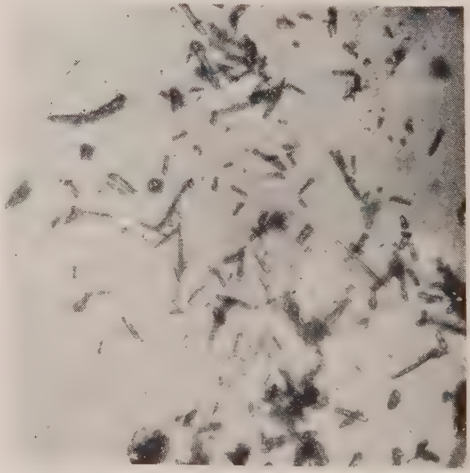

Fig. 3. Isoquercitrin from Bracken (from hot water) × 120

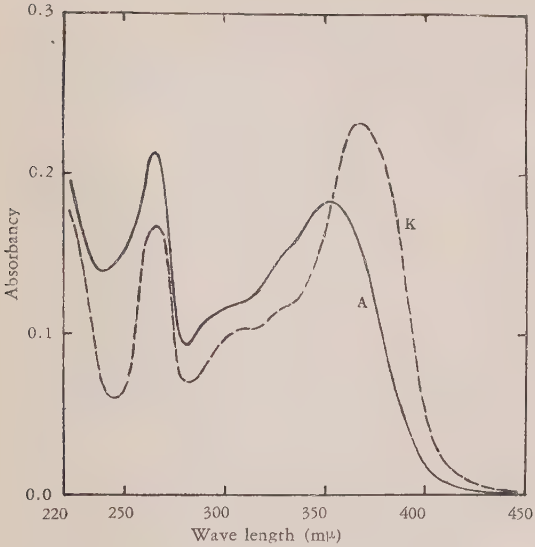

Fig. 4. The Spectrum of Astragalin and Kaempferol from Bracken
(10^{-5} M in ethanol solution)
A: Astragalin K: Kaempferol

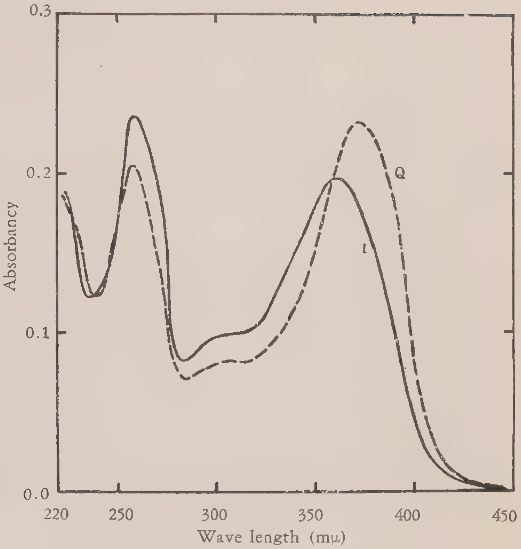

Fig. 5. The Spectrum of Isoquercitrin and Quercetin from Bracken
(10^{-5} M in ethanol solution)
I: Isoquercitrin Q: Quercein

The *R_F* value of this pigment agreed with one of isoquercitrin of the paper chromatogram (Table I. vi). It melted at 241~243° (uncorr.) and showed no depression on the mixed melting point determination with known isoquercitrin.

Anal. Found: H, 4.76; C 51.39, Calcd. for $C_{21}H_{20}O_{12}\cdot 1\frac{1}{2}H_2O$: H, 4.72; C 51.33%.

When hydrolyzed with 5 per cent sulphuric acid, the glycoside yielded quercetin and glucose which were identified by the paper chromatography (Table I. vii. viii). The recrystallized aglycone from alcohol was fine and yellowish needle, m.p. over 300°. Its acetylation gave colorless needle, m.p. 192~195° (uncorr.), which was identified as pentaacetyl quercetin from the analytical data and by its mixed melting point with an authentic specimen.

Anal. Found: CH_3CO, 41.81. Calcd. for $C_{15}H_5O_7(COCH_3)_5$: CH_3CO, 42.00%.

The absorption spectra of isoquercitrin and quercetin isolated from bracken were estimated and found to be identical with those of known pigments (Fig. 5). The maxima of these spectra in ethanol are as follows: Isoquercitrin... 258mμ, 362mμ; Quercetin... 258mμ, 372mμ.

IV. On the other Pigments: The reddish mother liquor separated from the above-mentioned two crystals was washed several times with ether, in which glycosides were entirely insoluble. This yellowish ether extract was distilled off and the residue, from which no crystalline pigment could be obtained, was dissolved in small quantity of ethanol. The flavonoid pigments in the ethanol solution and residual liquor were investigated by the method of the paper chromatography (Table I. ix, x).

On the paper chromatogram of the residual liquor after ether extraction, there were found two spots, corresponding to astragalin and isoquercitrin, plus an additional spot of the flavonoid pigment. For the reason of the agreement of its *Rf* value and coloration, it was evident that the position of this latter one corresponded to rutin.

The yellowish ethanol solution gave two spots of flavonoid pigments on the paper chromatogram and showed positive reactions of flavone, that is: first, dark greenish coloration by adding ferric chloride; secondly, reddish coloration in both cases when reduced with conc. hydrochloric acid and magnesium, and conc. hydrochloric acid and

Table I
Paper Chromatography of Flavonoid Pigments in Bracken

obtained	R_F value (color)						known	R_F value (color)	
	I			II				I	II
i. alc. ext. of fresh bracken	0.80	0.70	0.60	0.71	0.58	0.46			
ii. crude crystals	0.80(LY)	0.68(OY)		0.73(LY)	0.57(OY)				
iii. hot water slightly soluble crystals	0.78(LY)			0.73(LY)			astragalin	0.79(LY)	0.72
iv. aglycone of iii	0.83(Y)			0.62(Y)			kaempferol	0.84(Y)	0.63
v. sugar of iii	0.24(B)			0.36(B)			glucose	0.24(B)	0.35
vi. hot water soluble crystals	0.69(OY)			0.55(OY)			isoquercitrin	0.69(OY)	0.56
vii. aglycone of vi	0.70(OR)			0.27(OR)			quercetin	0.69(OR)	0.27
viii. sugar of vi	0.25(B)			0.36(B)			galactose	0.24(B)	0.42
ix. residual mother liquor	0.79(LY)	0.69(OY)	0.59(OY)	0.72(LY)	0.57(OY)	0.48(OY)	rutin	0.59(OY)	0.48
x. ether soluble fraction	0.88(PY)	0.82(Y)		0.64(PY)	0.41(Y)		quercitrin	0.80(OY)	0.60

Developing solvent ··· I: *n*-BuOH: AcOH: H_2O (4:1:2)
II: 80% Phenol
Color of spot ········Y: yellow O: orange R: red L: light P: pale B: brown
Color reagent ······· for pigments: 90% methanol solution saturated with basic lead acetate
for sugars: 90% ethanol solution containing benzidin, acetic acid, and trichloracetic acid

zinc, and thirdly, yellowish precipitate by adding lead acetate solution. These ether soluble pigments belonging to flavone could not be clarified still more, because the R_F values and coloration of these pigments were identical with none of the known specimens, such as kaempferol, quercetin, morin, myricetin, fisetin, astragalin, robinin, quercitrin, isoquercitrin, rutin, myricitrin, katuranin, distylin, amperoptin, fustin, astilbin, hesperetin, hesperidin, and the like, which had been already isolated from several plants by present author.

Since the colored spots of rutin and unknown two pigments on the paper chromatogram had small area and faint coloration, it was presumed that their quantity in bracken was less than astragalin and isoquercitrin.

V. Operation of Paper Chromatography: The operation of the abovementioned paper chromatography of flavonoid pigments is as follows: Toyo filter paper No. 50, 2 cm. wide and 40 cm. long, was used throughout. *n*-Butanol-acetic acid-water (4 : 1 : 2 by volume) and 80 per cent phenol by weight were used as the developing solvents. In most cases, the ascending method was adopted and the time required by two solvents for running 25∼30 cm. distance above the initial spots was 15∼20 hours at ordinary temperature. After the development was ready, the paper was dried to remove any trace of solvent and the position of pigments on the chromatogram was shown by spraying the dried paper with 90 per cent methanol solution saturated with basic lead acetate or 0.2 per cent ferric chloride in 90 per cent methanol solution. The position of sugars obtained when hydrolyzing the glycosides was shown by spraying 90 per cent ethanol solution containing benzidin, acetic acid, and trichloracetic acid. The R_F values and coloration of the flavonoid pigments and sugars by this method are shown in Table I.

VI. Estimation of the Thiamine-decomposing Activity of Flavonoid Pigments: For studying the thiamine-decomposing activity of flavonoid pigments, the method reported by Okamoto[11] was employed. The outline of the operation is as follows: The properly diluted pigment solution is incubated at 70° for one hour at pH 7.0 with 10 γ of thiamine in a total volume of 10 cc. The pigments are used in final concentration of 1 γ in 10 cc. The remaining thiamine is estimated by means of the thiochrome method by using Permutit. The thiamine-decomposing activity of pigment is expressed by the quantity of thiamine (γ) decomposed by 10 γ of pigment. The results obtained from the abovementioned experiments are shown in Table II.

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[11] T. Okamoto, *ibid.*

Table II

Thiamine Decomposing Activity of Several Flavonoid Pigments

(The activity is expressed by the quantity of thiamine (γ) decomposed by 10γ of pigment)

pigment	activity	pigment	activity
kaempferol	0.4	quercetin	4.4
astragalin	0.3	〃 + Mn^{++}	5.8
morin	0.7	isoquercitrin	3.5
hesperidin	0.8	rutin	2.7
myricetin	1.7	D-catechin	4.6

As shown in Table II, the thiamine-decompsing activity of kaempferol and its glycoside, like astragalin, is small but that of quercetin and its glycoside, such as isoquercitrin and rutin, is great and Mn^{++} accelerates the activity of quercetin. Moreover hesperidin and morin have small activitity but D-catechin shows great activity. These results show that flavonoid pigments possessing free orthodihydroxyl group in the side phenyl radical, like quercetin, its glycoside, and D-catechin, have the marked thiamine-decomposing activity. It is, therefore, supposed that isoquercitrin and rutin are a portion of the SF of bracken.

Summary

In order to make clear the so-called thermostable antithiamine factor of bracken, the properties of flavonoid pigments therein have been studied. The results obtained are as follows:

1. From the ethyl acetate soluble fraction of alcoholic extract of bracken, astragalin (kaempferol-3-glucoside) and isoquercitrin (quercetin-3-glucoside) were isolated in the form of yellow and needle crystals respectively.
2. In addition, rutin and two unknown flavonoid pigments were detected by the paper chromatography.
3. Among these pigments, isoquercitrin and rutin have the marked thiamine-decomposing activity.
4. It is, therefore, presumed that a portion of the thermostable antithiamine factor of bracken is the flavonoid pigments such as isoquercitrin and rutin therein.

Acknowledgements

The author wishes to express his heartfelt thanks to Prof. Y. Ōshima, Biochemical Laboratory of Kyushu University, for his kindness throughout these experiments and to Prof. A. Fujita, School of Medicine, Kyoto Prefectural University, for his advice.

Studies on Sinomenine.

Part LXVIII. (+)-*Codeinone from Sinomenine.*

By Kakuji GOTO and Izuru YAMAMOTO

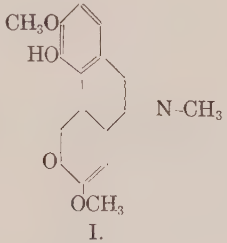
The Kitasato Institute, Tokyo, Japan

Received March 11, 1955

A short survey of sinomenine investigation is given in the theoretical part, principally on the transformation of sinomenine derivatives, hitherto almost unnoticed in morphine derivatives. In experimental, the preparation of (+)-codeinone from sinomenine, reduction of 1-bromocodeinone and similar compounds with $SnCl_2+HCl$, and the use of $NaBH_4$ on the reduction of the ketonic group in sinomenine derivatives are described.

Theoretical (K. G.)

Sinomenine (I) is the principal alkaloid of *Sinomenium acutum Rehd et Wils.* (Menispermaceae), a native climbing plant of south Japan. Its constitution was elucidated by the study of H. Kondo and E. Ochiai[1] on one side and of K. Goto[2] on the other, though its synthesis[3] was not yet realized. From sinomenine the following optical antipodes of morphine group were prepared and many of them[4] were well racemized with those corresponding substances derived from thebaine and codeine.



I.

(1) (+)-Dihydrothebainone
(2) (+)-Dihydrothebainol
(3) (+)-Tetrahydrodesoxycodeine
(4) (+)-7-Oxy-dihydrothebainol
(5) (+)-1-Bromosinomeninone
(6) (+)-α-Dihydrosinomeninone[4a]
(7) (+)-β-Dihydrosinomeninone
(8) (+)-Tetrahydrosinomeninone
(9) (+)-and (−)-7-Oxy-dihydrocodeine
(10) (+)-Desoxycodeine-D
(11) (+)-Dihydrocodeinone
(12) (+)-Dihydrocodeine[4b]
(13) (+)-Dihydromorphine[4b]
(14) (+)-Codeine
(15) (+)-Codeinone
(16) (+)-Morphine

The work to prepare (+)-meta-thebainone, (+)-thebenine and (+)-morphothebaine is now going on in the author's laboratory.

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[1] H. Kondo and E. Ochiai: *Ann.* **470**, 224 (1929) and others papers in *J. pharm. Soc. Japan* (1929-1930).

[2] 68th communications up to present, many of which are cited in this synopsis.

[3] Robert Robinson: *Madrid Lecture.*

[4] In some cases, the preparation of (−)-substance was renounced, on account of the shortage of materials.

[4a] K. Goto and Y. Shibasaki: *Ann.*, **503**, 277 (1933).

[4b] K. Goto and Tatsuo Arai: *Ann.*, **547**, 194 (1941).

As the detailed description of the determination of the constitution of sinomenine was reported in our former papers, here we wish to limit ourselves to survey the principal results obtained in our research and those reactions which were not hitherto observed by the other workers in morphine group.

From Sinomenine to (+)-Morphine

Since the establishment of the fact that sinomenine is an optical antipode of morphine group, our main object of the study was to derive (+)-morphine from sinomenine and to study its physiological properties.

The elemental synthesis of (−)-morphine was accomplished by Gates and his co-workers[5] in 1952, but it did not daunt our long cherished project. As our former trials to reach (+)-morphine from several sides (including from (+)-dihydrothebainone) were all fruitless, we took up Gates's method, which used 2,4-dinitrophenylhydrazine to fix the ketone group of the brominated dihydrothebainone. Our method[6] modified that of Gates in several minor points and was shown in the following diagram.

Sinomenine $\xrightarrow{\text{Cat. red.}}$ Dihydrosinomenine
$\xrightarrow{\text{Na-Hg}}$ (+)-Dihydrothebainone $\xrightarrow{+3Br_2}$
1,5,7-Tribromo-(+)-dihydrothebainone → 2,4-dinitrophenylhydrazone $\xrightarrow[\text{and cleavage}]{\text{Pyridine (heat)}}$ 1-Bromo-(+)-codeinone $\xrightarrow{LiAlH_4}$ (+)-Codeine
$\xrightarrow{\text{Pyridine-HCl}}$ (+)-Morphine

Or alternatively,

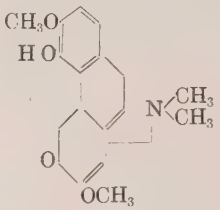
(+)-Dihydrothebainone $\xrightarrow[\text{Alkali}]{+2Br_2}$ (+)-1-Bromo-dihydrocodeinone $\xrightarrow{+Br_2}$ 1,7-Dibromo-(+)-dihydrocodeinone → 2,4-dinitrophenylhydrazone
$\xrightarrow[\text{and cleavage}]{\text{Pyridine(heat)}}$ (+)-1-Bromo-codeinone

The over-all yield of (+)-morphine from sinomenine was 0.2% at best. A better method must be devised to prepare enough material in order to proceed to its pharmaceutical study.

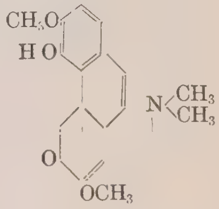
Three Sinomenine-methines[7]

Sinomenine gives three kinds of methine, viz.,

color reaction with conc. H_2SO_4
(1) Sinomenine achromethine (II) almost colourless
(2) „ roseomethine (III) red
(3) „ violeomethine (IV) deep blue



II.



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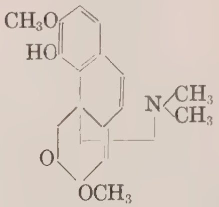

IV.

Achromethine is formed, when sinomenine methiodide is boiled with the calculated quantity of 2% NaOH for one minuite. A longer boiling turns it into roseomethine. As achromethine takes no colouration with conc. H_2SO_4, the author assumes its double bond to be in $C_9 \sim C_{14}$. Achromethine is turned on long keeping into roseomethine, whose second double bond is supposed to be between $C_9 \sim C_{10}$, because of its red colour reaction with conc. H_2SO_4. Keeping achromethine in

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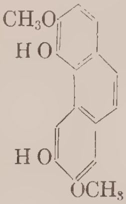
[5] M. Gates and G. Tschudi: *J. Am. Chem. Soc.*, **74**, 1109 (1952).

[6] K. Goto and I. Yamamoto: *Proc. Japan Acad.*, **30**, 769 (1954).

[7] K. Goto and H. Shishido: *Bull. Chem. Soc. Japan*, **6** 76 (1931).

10% KOH overnight turns it into violeomethine, perhaps the two double bonds being at $\Delta^{8,14}$ and $\Delta^{9,10}$.

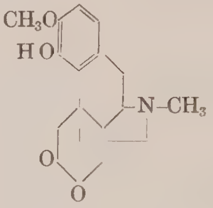
As to the colouration of substances with conc. H_2SO_4, there are studies of Schöpf[8] on meta-thebainone and of Kuhn[9] on diphenyl-polyene. Author's experience shows also that all des-N-methyl-bases of sinomenine derivatives give red colouration with conc. H_2SO_4, but their dihydrodes-N-methyl-bases do not. Well known red colouration of thebenine with conc. HCl disappears[10], when it is hydrogenated on its $\Delta^{9,10}$. Those derivatives of sinomenine, whose conjugated double bonds are assumed to be also conjugated with benzene nucleus take blue colour with conc. H_2SO_4.


V.

Sinomenine or its methiodide is decomposed into sinomenol, namely, 3,7-dimethoxy-4,6-dioxy-phenanthrene (V), by boiling with 66% caustic soda for one hour[11]. The liberated amine is methyl-ethyl- resp. dimethyl-ethyl-amine.

Sinomeninone and Sinomeninic acid

Sinomenine is easily hydrolyzed on its enol-methoxyl and gives sinomeninone (VI)[12]. Sinomeninone, an α-diketone, is again easily oxidized by 30% H_2O_2 into sinomeninic acid[13], in which hydrolyzed ring (III) of sinomenine is opened. By catalytic reduction with $PdCl_2$, sinomeninone gives α- and β-dihydrosinomeninones, α- being 6-keto-7-ol (VIII) derivative and β- being 7-keto-6-ol (IX) substance. This relation was well established by the hydrolysis of sinomeninol (X), which was obtained by $LiAlH_4$ reduction of sinomenine and gave exclusively β-dihydrosinomeninone[14]. On the contrary, the both ketone groups were reduced by PtO_2 as catalyst and tetrahydrosinomeninone was formed. By boiling tetrahydrosinomeninone with 55% sulphuric acid, a new (+)-dihydrothebainone was isolated, which was totally different from the known (+)-dihydrothebainone and we regarded it as (+)-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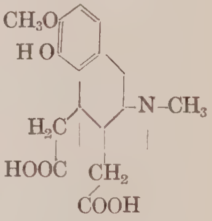
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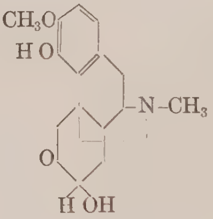
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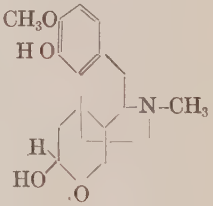
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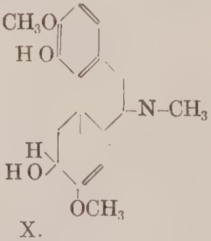
X.

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[8] C. Schöpf and Borkowsky: *Ann.*, **458**, 148 (1927).

[9] R. Kuhn: *Helv. Chim. Acta.*, **13**, 64 (1930).

[10] K. Goto, H. Shishido and K. Takubo: *Ann.*, **497**, 295 (1932).

[11] K. Goto, H. Sudzuki: *Bull. Chem. Soc. Japan.*, **4**, 163 (1929).

[12] K. Goto and H. Sudzuki: *Bull. Chem. Soc. Japan.*, **4**, 271 (1929).

[13] K. Goto, K. Takubo and S. Mitsui: *Ann.*, **494**, 1 (1932)

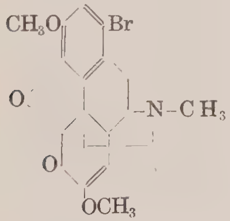
[14] K. Goto, I. Yamamoto: *Proc. Japan Acad.*, **29**, 513 (1954).

dihydrothebainone-7[15].

Sinomeninic acid, its derivatives and (−)-tetrahydrosinomeninone were also obtained from (−)-1-bromosinomeninone and were racemized with corresponding (+)-derivatives.

1-Bromosinomeneine

This substance was prepared by the bromination of sinomenine with two molecules of bromine. By elemental analysis, it was proved that it contained only one atom of bromine and at the same time three hydrogen atoms less than sinomenine[16]. While the author was reserving a decision on its constitution, C. Schöpf succeeded to close the oxide ring in (−)-dihydrothebainone by bromination and suggested that the 1-bromosinomeneine must have the oxide ring closed. The author, hereupon, took out, by its decomposition with dimethyl sulphate and alkali, a morphenol (XVII) derivative, instead of morphol derivative and verified Schöpf's suggestion[17]. 1-Bromosinomeneine, as an optical antipode of hypothetical 1-bromo-7-methoxy-(−)-codeinone, shows many peculiar characters, some of which will be reported here shortly.



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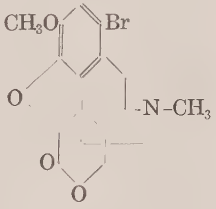
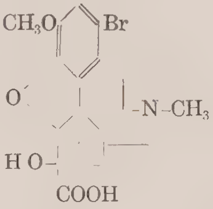


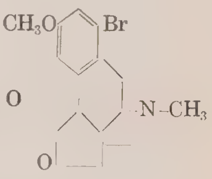
XII.

Sinomenilic acid ; Naphtindene alkaloids

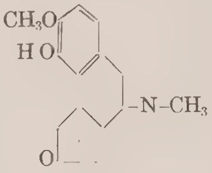
1-Bromosinomeneine is also hydrolyzed into 1-bromosinomeneine ketone (XII)[17] in the same treatment as with sinomenine. But this epoxy-6,7-diketone undergoes very easily benzilic acid transformation, when it comes in contact with cold, dilute caustic alkali. The here obtained 1-bromosinomenilic acid loses formic acid by cold fuming sulphuric acid and is transformed into 1-bromosinomenilone[18]. The debrominated substance is a new type of alkaloids, which has a naphtindene skeletone[19]. The oxide ring of sinomenilone is opened by sodium amalgam and gives dihydrosinomenilone (XV), which in turn was decomposed by Hofmann's method into a thebenone analogue. But, this nitrogen free substance, in the way of preparation, loses one molecule of water from its two molecules and is condensed into anhydro-bis-sinomelone, which was strongly laevorotatory.



XIII.



XIV.



XV.

The ketone oxygen of dihydrosinomenilone was replaced by two chlorine atoms and then by two hydrogen atoms. The obtained dihydrosinomenilane is also decomposed into sinomelane and dihydrosinomelane (monomolecular)[20].

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[15] K. Goto and K. Michi: *Bull. Chem. Soc. Japan*, **22**, 262 (1949)

[16] K. Goto and T. Nambo: *Bull. Chem. Soc. Japan*, **5**, 165 (1930).

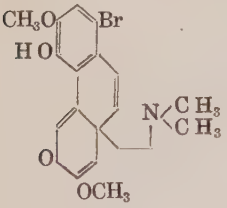
[17] K. Goto, K. Takubo and S. Mitsui: *Ann.*, **489**, 86 (1931).

[18] K. Goto, H. Shishido and K. Takubo: *Ann.*, **495**, 122 (1932).

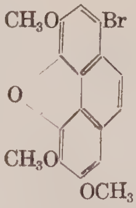
[19] K. Goto and K. Takubo: *Ann.*, **499**, 169 (1932).

des-N-Methyl-1-bromo-dehydro-meta-sinomenine

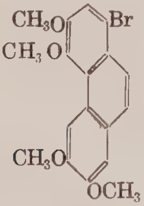
The second remarkable disintegration of 1-bromosinomeneine was found in its methiodide[21]. When the latter was treated with cold, 0.2% caustic soda, it was transformed momentaneously into des-N-methyl-1-bromo-dehydro-meta-sinomenine (XVI). When we carried out this reaction with 10% NaOH at 100°, we obtained a cinnabar red Na-salt of the oxy-quinone. This des-N-methyl-base was decomposed by boiling caustic soda solution into 1-bromosinomenol, namely 1-bromo-4,6-dioxy-3,7-dimethoxy-phenanthrene quantitatively. This shows that the ethanamine chain must be attached to C_{13} or C_{14} in this substance. In the oxy-quinone formula this side chain could not stand at C_{13}, and we assumed its location at C_{14}. This easy disintegration reminded us of Knorr's observation that methiodide of codeine was too labile to be recrystallized from water[22].



XVI.



XVII.



XVIIa.

This disintegration explains clearly the fact that in the Hofmann decomposition of 1-bromosinomeneine with dimethylsulphate and caustic soda, we sometimes obtained morphenol (XVII) and sometimes morphol (XVIIa). If the above transformation happened before the decomposition we obtained only morphol derivative, but when the ketone group at C_6 was first transformed into enol-methoxyl, then the oxide ring would be kept intact and we obtained morphenol derivative.

The last fact induced us to try Hofmann decomposition of sinomenine derivatives with dimethyl sulphate and alkali at lower temperature (70°). We obtained 1-bromosinomenol dimethyl ether from 1-bromosinomenine in a good yield, but sinomenine itself gave bimolecular phenanthrene, which was different from the known disinomenol dimethyl ether, perhaps two molecules having been linked together at C_5, in an ortho position to a newly formed phenol group. This linking of oxy-phenanthrene in para or ortho position by warming with dilute caustic alkali, seems to be a general reaction. Pure sinomenine gives in the alkalysis with 66% NaOH, a small quantity of disinomenol. Acetyl thebaol gives bis-1,1′-dithebaol in 45% yield, when warmed with 10% alkali on a water bath while stronger alkali (50% KOH) had no similar effect on it[23].

1-Bromosinomeneine alcohol

By Meerwein-Pondorf reduction of 1-bromosinomeneine, we obtained 1-bromosinomeneine alcohol (XVIII)[24]. The fact that in the latter substance the original ketone group was reduced to a secondary alcohol was proved by the reformation of the 1-bromosinomeneine through its oxidation by CrO_3 or $KMnO_4$ and by Oppenauer's method. One very remarkable property of this alcohol is that, when it comes in contact with cold dilute hydrochloric acid, it is transformed into 1-bromosinomeninone (XIX) in a few minutes. This may be explained by the hydrolysis of enol methoxyl, α-ketol-transformation and then opening of the oxide ring. We often experienced that, in the alkaloids of sinomenine type with a ketone group on C_6, an oxide ring and a hydroxyl or methoxyl on C_7 can not co-exist, and they are converged into one ketone group on C_7. Thus, dihydrosinomenine can not close the oxide ring by dibromination, but gives 1-bromosinomeninone. This fact is rather contrasted with the stability of 1-bromosinomeneine ketone, 7-oxy-dihydrocodeine and 7,8-dioxy-dihydrocodeine. Anyhow, the above mentioned transformation is very remarkable, because such a complex reaction is accomplished by such a mild reagent so rapidly.

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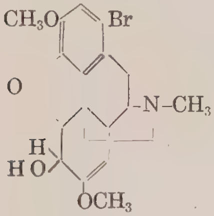
[20] K. Goto and H. Shishido: *Ann.*, **507**, 296 (1933).

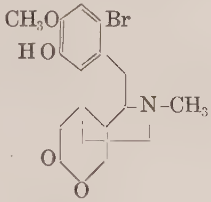
[21] K. Goto, T. Arai and T. Odera: *Bull. Chem. Soc. Japan*, **17**, 393 (1942).

[22] Ach, Knorr: *Ber.*, **36**. 3067 (1903).

[23] K. Goto, T. Arai and T. Odera: *Bull. Chem. Soc. Japan*, **18**, 116 (1943).



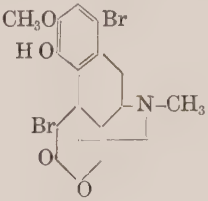
XVIII.



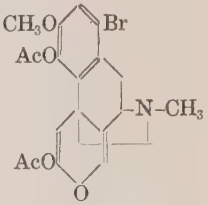
XIX.

Acetolysis of 1-Bromosinomeneine ketone

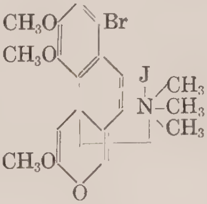
By boiling with acetic anhydride, 1-bromosinomeneine ketone or 1,5-dibromosinomeninone (XX) is decomposed into 1-bromo-3-methoxy-4,6,7-triacetoxy-phenanthrene and diacetyl-1-bromodehydrosinomeninone[24a] (XXI). The latter substance was isolated as methiodide (the free base was not crystallizable). This methiodide was decomposed into des-N-methy-l-bromo-dehydrosinomeninone by mild treatment with caustic alkali. This des-N-methyl-base contains only one optical centre at C_{13} and is strongly dextrorotatory, $[\alpha]_D=+283.9°$. By the treatment of the diacetyl methiodide with dimethyl sulphate, alkali and potassium iodide, we obtained methiodide of des-N-methyl-1-bromo-dehydro-sinomeninone dimethyl ether. This substance, as well as des-N-methyl-1-bromo-dehydrosinomeninone, gave deep blue colour with conc. sulphuric acid. This colour reaction seems to be characteristic in sinomenine derivatives when two double bonds are conjugated to the benzene nucleus. We assume, therefore, a new double bond, which was introduced by fission of the oxide ring, was shifted to $C_{8\sim14}$. If this assumption is true, it is noteworthy that such a shifting occurred over the ethanamine chain. It is only explicable by assuming an intermediate three or



XX.



XXI.



XXII.

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24) K. Goto, T. Arai and T. Kono: *Bull. Chem. Soc. Japan*, **23** 17 (1950).

24a) K. Goto, R. Mori and T. Arai: *Bull. Chem. Soc. Japan* **17**, 439 (1942).

four membered ring formation. The above methiodide of des-N-methyl-dimethyl ether gave by acetolysis a good yield of 1-bromo-7-acetoxy-3,4,6-trimethyl-phenanthrene.

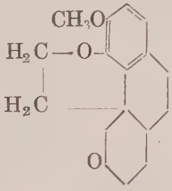
The reaction, here explained, was carried out also with (−)-1,5-dibromo-sinomeninone from thebaine. All the corresponding derivatives were racemized with those from sinomenine.

(−)-Thebenone; a new proposal

Thebenone (XXIII) was first prepared by Wieland and Kotake[25] by the Hofmann decomposition of (−)-dihydrothebainone. We have prepared five thebenones from sinomenine derivatives as shown in Table I.

Table I.

	(+)-2H-thebainone[26]	2H-sinomenine[27]	(+)-4H-desoxycodeine[28]	(+)-1-Br-2H-thebainone[29]	Sinomeninone-furazane[28]
Base	+ 59°	+194°	+ 43°	+ 79°	+136°
des-N-base	− 55°	− 84°	− 65°	− 8°	+ 50°
2H-des-N-base	+ 68°	+ 2°	+ 78°	+ 61°	+ 22°
Dehydrothebenone	−207°	−286°	−178°	−186°	−485°
Thebenone	− 79°	−148°	− 3°	− 23°	−120°



XXIII.

It is noteworthy that the optical rotation changes its sign stepwise from base to thebenone. Thebenone from sinomenine (+) is laevorotatory, while thebenone from thebaine (−) is dextrorotatory. Here some confusion on the original substance occurrs, if we simply designate them as (+) or (−)-thebenone. We propose therefore in the study of sinomenine or morphine, we should prefix D to the derivatives of sinomenine and L to those of morphine group in necessary case. Thus if we designate thebenone from sinomenine as D-thebenone (−) and that from thebaine as L-thebenone (+), there could be no confusion. Further examples will be furnished later.

This nomenclature was already adopted in cases of amino acids and monosaccharides. We hope this proposition would be taken into consideration by organic chemical circles.

(+)-True Thebainone

Sinomenine is laevorotatory. But when its double bond is reduced, it becomes invariably dextrorotatory. These dextrorotating derivatives are always the optical antipodes of morphine derivatives, if in the latter series the corresponding compounds exist. From this fact, it is beyond doubt that the skeleton of sinomenine is the optical antipode of that of morphine group. The laevorotation of sinomenine seemed therefore to be caused by its double linking[30].

In 1931, C. Schöpf[30] prepared true thebainone (XXIV) from thebaine. L. Small[31] and K. Goto[32] found that it was laevorotatory. This showed that if we

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25) H. Wieland and M. Kotake: *Ann.*, **444** 88 (1925).

26) K. Goto, R. Inaba and H. Shishido: *Ann.*, **485**, 247 (1931).

27) K. Goto and H. Shishido: *Bull. Chem. Soc. Japan*, **6**, 231 (1931).

28) K. Goto and S. Mitsui: *Bull. Chem. Soc. Japan*, **6**, 197, (1931).

29) K. Goto, H. Ogawa and J. Saito: *Bull. Chem. Soc. Japan*, **10**, 481 (1935).

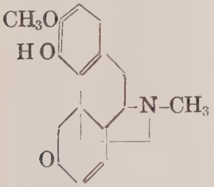
30) C. Schöpf and H. Hirsch: *Ann.*, **489**, 244 (1931).

31) L. Small and D.E. Morris: *J. Am. Chem. Soc.*, **54**, 2122 (1932).

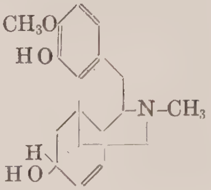
32) K. Goto and H. Ogawa: *Ann.*, **511**, 202 (1934).

could prepare 7-demethoxysinomenine, it must turn the plane of polarization to right. K. Goto and I. Yamamoto[33] prepared recently 7-demethoxysinomenine from α-dihydrosinomeninone and found that it was dextrorotatory and well racemized with Schöpf's true thebainone. The laevorotation of sinomenine seems, thus, not to be caused by double linking, but caused by the enol-methoxyl attached to the double bond. Such action of methoxyl which stands in β- or γ-position to the optical centre, on the invertion of optical rotation is, we think, rather noteworthy.

We can add one more instance, hitherto to have been met with, in which sinomenine derivative showed laevorotation. The 7-demethoxysinomeninol (XXV) which was prepared by $LiAlH_4$ reduction of 7-demethoxysinomenine[34] showed laevorotation, and racemized with (+)-thebainol from true thebainone. Here the rotation is inverted in morphine and sinomenine group. This is one of the reasons in our proposal to prefix their names with L- or D- in necessary cases.



XXIV.



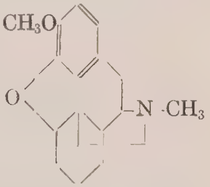
XXV.

Two new ring closures

(1) When α-dihydrosinomeninone (VIII) was boiled with 50% sulphuric acid at 130° for one hour, we obtained (+)-dihydrocodeinone in 50% yield[35]. Dihydrosinomenine behaved also perfectly in the same way. This remarkable reaction may be explained by the anionotropy of hydroxyl group, which stands in α-position to ketone. If we assume the ketone group enolized, then this anionotropy is similar to the geraniol-linalool transformation. R. Robinson explained the codeine-pseudocodeine transformation on the same basis[36].

β-Dihydrosinomeninone gave (+)-dihydrocodeinone also in the same way. In this reaction α-ketol transformation must preceed the anionotropy.

XXVI.



(2) When (+)-dihydrothebainol was treated in the same way, the oxide ring was also closed and we obtained (+)-dihydrodesoxycodeine-D (XXVI). This reaction seems to have been brought about by the introduction of a double bond between $C_5 \sim C_6$ and then the addition of phenol group to this double linking.

Bimolecular alkaloids of Disinomenine type

Disinomenine (XXVII) was first isolated by the spontaneous decomposition of the gold chloride double salt of sinomenine hydrochloride and afterwards from the plant itself. The alkaloid is bimolecular and the linking position is assumed to be in 1,1′, because of the strong decrease of the diazo-reaction of sinomenine, which is still noticeable in 2,000,000th dilution with diazobenzene sulphonic acid. Other alkaloids of sinomenine type (OH in 4, H in 1) can be linked together in the same way with mild oxidizing agents, such as gold chloride, silver nitrate, ferric chloride, potassium ferri-cyanide and alkali, dilute permanganate and dilute hydrogen peroxide. The linking was tried with the following seven alkaloids and we obtained always a pair of the bimolecular alkaloids. The relation of these alkaloids are shown by arrows in Table II.37)

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[33] K. Goto and I. Yamamoto: *Proc. Japan Acad.*, **29**, 210 (1953).

[34] K. Goto and I. Yamamoto: *ibid.*, **29**, 457 (1953).

[35] K. Goto and K. Michi: *Acta Phytochimica* (**1949**) 183, 187.

[36] Gulland and Robinson: J. Chem. Soc., 123, 980 (1923).

Table II.

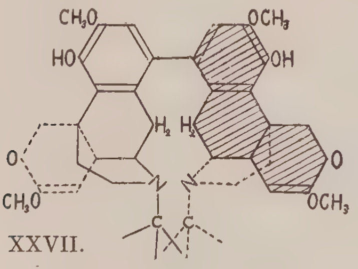
Linking of	Bis-1,1′-derivatives		
	normal		pseudo
1) Sinomenine	normal ↓		pseudo ↓
2) Dihydrosinomenine	←normal— ↓	Na-Hg	pseudo——→ ↓
3) Dihydrosinomeninol	normal		pseudo
4) (+)-Dihydrothebainone	←normal← ↓		amorphous →
5) (+)-Dihydrothebainol	normal		....
6) (+)-Tetrahydrodesoxycodeine	→n-JCH$_3$	base mixture	φ-JCH$_3$ ←——
7) meta-Thebainone	normal		pseudo

Note 1) Short, vertical arrows show catalytic reduction.
2) Long arrows on both sides indicate Clemmensen reduction.
3) Normal series forms crystallizable hydrochloride, but pseudo series does not.
4) Bis-1,1′-sinomenine, whose hydrochloride is crystalline, is natural and we call this series normal.

As to the cause of the existence of these pairs, we came now to the assumption that it is a similar, but a particular case of the stereoisomerism of the substituted diphenyls. Only the difference is that in this case the hindering facter of free rotation is the ethanamine chains, instead of ortho substituents of diphenyl nucleus. In linking of these alkaloids, two molecules can be linked together in two ways, namely in one case *en face* and in the other case *en dos*. In other words, the two alkaloids are only different in the way of overlapping of the ethanamine chains (I and II).

A————·······B A·······————B
I II

But when the formula I wants to become II, one of the component must rotate ca. 360 degrees (in extreme case) and we imagine that this farreaching free rotation is greatly hindered by some reason hitherto unknown. This assumption seems to have been proved partly by the Hofmann decomposition. n- and φ-bis-1,1′-(+)-tetrahydrodesoxycodeine gave the same bis-1,1′-des-N-methyl-base, the same bis-1,1′-dehydrothebenane and the same bis-1,1′-thebenane. The identity of respective derivatives from both sources was proved by the m.p., specific rotatory power and ultraviolet absorption. The same was true of the bis-1,1′-dihydrosinomenine.



XXVII.

Bis-1,1′-sinomenines and bis-1,1′-metathebainones38) are not fitted for this decomposition, as they gave the same bimolecular phenanthrene respectively. But the fact that only one form is

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37) K. Goto, I. Yamamoto and S. Mastumoto: *Proc., Japan Acad.*, **30**, 883 (1954); *Bull. Agr. Chem. Soc. Japan*, **19**, 1 (1955).

38) K. Goto and Z. Kitasato: *Ann.* **481**, 81 (1930).

hitherto known in pseudomorphine seems to be of value in our argument. If it is linked together in 2,2 -position as we assume, the overlapping difference of ethanamine chains can not occurr.

Experimental (I. Y.)

(1) **2,4-Dinitrophenylhydrazone of (+)-1-bromocodeinone.**

(+)-Dihydrothebainone (2 g.) was brominated (3.2 g. Br_2 ; 3 mol.) in glacial acetic acid (20 cc). To this solution, dinitrophenylhydrazine (1.44 g ; 1.1 mol.) was added. After the latter dissolved, fused sodium acetate (1.1 g ; 2 mol.) was added to fix free hydrogen bromide. The whole was then incubated at 28° for 20 hours and the acetic acid was removed i.v. at 50°. Seven lots of the residue were united and boiled with 140 cc of pyridine for 30 min. (bath temp. 130–140°). Pyridine was distilled i.v. and the residue was taken up in much chloroform. The chloroform was washed many times with 10% NaOH, and the remaining pyridine was removed by washing with 10% HCl. The dinitrophenylhydrazone remained in chloroform in this operation. The chloroform was washed with soda, dried and concentrated and passed through a column of Al_2O_3. The elution was done also with chloroform. From the residue of chloroform evaporation, the required dinitrophenylhydrazone crystallized out on addition of ethyl acetate. Yield 47% of theory.

(2) **(+)-Codeinone.** As Oppenauer's oxidation of (+)-codeine into (+)-codeinone failed[39], we returned to the original method of Knorr (*Ber.*, **36**, 3067 (1903)). Yield 0.1 gr. from 0.8 gr. (+)-codeine. M.p. 185° after two recrystallizations from ether. $[\alpha]^{14}_{D}=+206.0^\circ$ (c 0.334, alc.). (*Anal*. Calcd. for $C_{18}H_{19}O_3N$: C, 72.70 ; H, 6.44 ; N, 4.71. Found : C, 72.49 ; H, 6.15 ; N, 4.79).

(3) **d,1-Codeinone.** 0.013 gr. each of (−)- and (+)-codeinone (m.p. 185° in both substances) were dissolved in ethyl acetate+ether and the residue of the evaporation of the solvent was twice recrystallized from acetone. M.p. 175°, $\alpha=\pm0^\circ$ (c 0.2, alc.).

(4) **Reduction of 1-bromosinomenine derivatives with $SnCl_2$ and HCl.**

Through reducing with stannous chloride and hydrochloric acid, (+)-1-bromocodeinone gave unexpectedly (+)-dihydrothebainone, instead of (+)-1-bromometathebainone. The reduction of nuclear halogen atom, which stands in ortho or para position to phenol group, was already reported by H. Burton (*J. Chem. Soc.*, **1945**, 280). But the influence of the bromine atom in (1) of bromo codeinone on the molecule as a whole is rather surprising.

We tried the same reduction with seven other 1-bromosinomenine derivatives and obtained similar results, as shown in Table III.

(5) Reduction of the ketonic group of sinomenine derivatives with $NaBH_4$. We tried the reduction with following seven substances and the results are summarized in Table IV.

Table III.

	Starting meterial (all (+))	Reduced substance obtained	Yield
1)	1-Bromocodeinone	Dihydrothebainone	30%
2)	1-Bromodihydrothebainone	”	53%
3)	Tribromodihydrothebainone	”	33%
4)	1-Bromosinomeneine	β-Dihydrosinomeninone	34%
5)	Sinomenine	”	57%
6)	1-Bromosinomeninone	”	31%

1) It was rather curious that we isolated only β-form and not α-form. It was proved that α-form was transformed into β-form by hot hydrobromic acid but the change was not complete (K. Goto and Y. Shibazaki : *Ann.*, **503**, 281 (1933). 2) Efficacy of $SnCl_2$ used in these experiments was proved by transforming (−)-codeinone into (−)-meta-thebainone (Yield ca. 30%).

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[39] However, Findlay and Small: *J. Am. Chem. Soc.* **73**, 4001 (1951).

Table IV.

	Starting Material (+)	Reduced Substance	Yield (%)	Alternative method
1)	Dihydrocodeinone	(+)–Dihydrocodeine	80	Cat. reduction with PtO_2+ H_2 in MeOH or Pyridine
2)	Bromosinomeneine	Bromosinomeneine alcohol	70	Meerwein-Pondorf's reduction
3)	Sinomenine	Sinomeninol	40	$LiAlH_4$ in 4H-furane
4)	Dihydrosinomenine	Dihydrosinomeninol	70	Cat. red. with PtO_2+H_2 or Na-Hg reduction
5)	Dihydrothebainone	Dihydrothebainol	70	Na-Hg reduction
6)	Sinomeninone	Tetrahydrosinomeninone	60	Cat. red. with. PtO_2+H_2
7)	Bromosinomeneine-ketone	a new substance of m.p. 165° (Yield 60%), which is free from ferric chloride reaction, resistant to 10% NaOH, but is transformed into Bromosinomeninone by 10%HCl. This substance is perhaps 1-bromo-4,5-epoxy-6-hydroxy-7-keto derivative.		

The general procedure of these experiments is as follows. The starting material is dissolved or suspended in 20 times methanol, and is added with excess of $NaBH_4$. Evolution of hydrogen is accompanied. After 2 hours ca. 2/3 of the methanol is evaporated and the base is isolated from the caustic alkaline solution (in case of non-phenolic base) or from the soda alkaline solution.

Note: The experimental part and a part of the theoretical of this paper were read by K. G. in the XIVth International Congress of Pure and Applied Chemistry, Zurich, July, 1955.

Studies on Sensitive Groups of Crystalline Bacterial α-Amylase

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Received March 30, 1955

Effect of Folin's phenol reagent, fluorodinitrobenzene, and iodine on crystalline bacterial α-amylase were investigated to ascertain the relationship between enzymic activity and chemical reactive groups of the enzyme.

From the results the tyrosyl groups of the enzyme protein were concluded to be essential to enzymic activity. A simplified method to prepare the bacterial α-amylase in crystalline form and some properties of the enzyme are also described.

Introduction

The present study was undertaken in connection with some works on the "amylase destroying enzyme"[1, 2, 3, 4] and aims to determine what chemical group is most intimately related to the enzyme activity of bacterial α-amylase.

The relationships between the chemical groups of α-amylase and the enzymatic activity have long been studied by several workers. Little and Caldwell[5] reported that primary amino groups are essential to the activity of pancreatic amylase, whereas the hydroxyl group of tyrosine seems to have no importance. Myrbäck and Frostell[6] assumed that primary amino groups are essential for the combination of malt α-amylase with substrate. From the kinetical study of mold amylase Myrbäck and Willsteadt[7] also concluded that basic groups in the uncharged state bind the substrate, and that acid groups in the uncharged form are in a certain sense indispensable to the enzyme activity.

In the present paper a simplified crystallization method of bacterial α-amylase was attempted and an investigation of the effect of phenol reagent, iodine, and 1-fluoro-2, 4-dinitrobenzene (FDNB) on the crystalline bacterial α-amylase was carried out to ascertain the relationship between the enzymatic activity and the tyrosyl, tryptophanal, and amino groups of the amylase. The results obtained were of special interest for two reasons: (1) the tyrosyl groups and tryptophanal groups, especially the former, seemed to be essential to the enzymatic activity of the amylase, contrary to previously known results for other α-amylases, and the chemical changes of these groups seemed to lead to inactivation without bringing about much changes in molecular structure of the amylase protein, and (2) a certain basic groups of the amylase protein seemed to be of no importance.

Experimental

Crystallization of Bacterial α-Amylase.

In the present study bacterial α-amylase (lique-

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[1] T. Yamamoto, G. Chiba, and J. Fukumoto, *Symposia on Enzyme Chem*. (Japan), **8**, 54 (1953).

[2] T. Yamamoto and J. Fukumoto, *Ibid*, **9**, 45 (1954).

[3] T. Yamamoto and J. Fukumoto, *Ibid*, **10**, 32 (1954).

[4] T. Yamamoto, This bulletin, **19**, 20 (1955).

[5] J. E. Little and M. L. Caldwell, *J. Biol. Chem.*, **142**, 585 (1942); *Ibid*, **147**, 229 (1943).

[6] K. Myrbäck und K. Frostell *Arkiv Kemi, Mineral, Geol.*, **24**A, No. 11, 11 pp (1946); *C. A.*, **42**, 6869 (1948).

[7] K. Myrbäck und E. Willsteadt, *Arkiv Kemi*, **3**, 443 (1951); *C. A.*, **46**, 7132 (1952).

fying type) of *Bac. amyloliquefaciens* Fukumoto[8),9)], a strain of *Bac. mesentericus* was used. Crystallization was carried out in the following manner:

The culture broth (submerged culture for 24 hrs. at 37°C in a medium containing soybean cake extracts, 5%; liquefied starch, 3%; $(NH_4)_2HPO_4$, 1.2%; $MgSO_4 7H_2O$ and KCl, 0.02%; pH 7.0) was filtered through Celite, and ammonium sulfate was added to the filtrate bringing it to 18% concentration by weight. After cooling the solution to 8°C, the supernatant solution (pH 6.0) was passed through a cornstarch layer (1g cornstarch per 7,000 LSV amylase activity) with an aid of a hard suction causing amylase to be adsorbed on the cornstarch. The cornstarch was then suspended in a phosphate buffer solution (M/50, pH 7.4), four times its volume, and allowed to stand for 1 to 2 hrs. at 30°C. Thereafter, ammonium sulfate was added to the supernatant solution to make up to 30% concentration. The resulting precipitate was collected by centrifuge and dissolved into M/200 calcium acetate solution equal in volume to the first elute of the phosphate solution, and after filtration, salted out again with ammonium sulfate. The precipitate thus obtained was dissolved in a small amount of distilled water, and the supernatant solution was dialysed against M/200 calcium acetate solution (pH 6.0) in the cold for several days changing the outer solution frequently. Usually, crystals were formed during dialysis, and addition of acetone to the dialyzed solution (ca. 10%) resulted in a rapid crystallization. The first crystals (activity recovery, ca. 80%) were collected and washed with distilled water by centrifugation. The washed crystals were then suspended in a small amount of distilled water and saturated solution of calcium hydroxide was added to it dropwise until the pH value reached about 10, where the crystals easily dissolved. The solution was neutralized with acetic acid to pH 6.0, and allowed to stand in the cold, then the amylase crystallized out again. The crystalline amylase in the present work was recrystallized seven times in this manner. By repeating recrystallization the crystals which were at first long needles varying in size transformed into somewhat rectangular forms (microscopically, ca. 8 × 1) of almost equal size.

Properties of the Crystalline Bacterial α-Amylase.

1. General Properties. The crystalline amylase contained 15.2% of nitrogen. The reducing sugar formation of the amylase in five minutes at 40°C was 12,100 mg in maltose equivalent (an aliquot of the following reaction mixture was analyzed by a modification of Fehling-Lehmann-Schoorl method[10)] and reducing sugar formed was calculated as maltose, mg: 10 ml. of 2% soluble starch, 9 ml. of M/30 acetate buffer (pH 5.8) and 1 ml. of enzyme solution) and starch liquefying power by the Lintner-Sollied method[11)] representing the amount (g) of potato starch liquefied in 15 min. was 13,200 (LSV), per mg of the protein nitrogen. Ultraviolet absorption spectra of the amylase are shown in Fig. 1. From the absorption coefficient of neutral and 0.1 N alkaline solution at 275mμ and 294mμ, the content of tyrosine and tryptophan[12)] in the amylase was calculated approximately to be 6.8% and 9.1%, respectively. In the color reaction with phenol reagent[13)] (amylase solution was heated in the presence of 0.1 N NaOH for 30 mins. at 100°C. After cooling and neutralizing with 0.1 N HCl to pH 9.0, 3 ml. of 20% Na_2CO_3 solution was added and the total volume was brought up to 9 ml. To this solution 0.5 ml. of the phenol reagent, diluted to ten times its volume, was added and the optical density was determined by photoelectro photometer after standing for 30 mins. at 25°C.) the amylase gave a color value corresponding to 10.7% as tyrosine. The ratio of the color value

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8) J, Fukumoto, *J. Agr. Chem. Soc. Japan*, **19**, 487, 634, 689, 789, 853 (1943); *Ibid*, **20**, 23, 121, 309 (1944).

9) The amylase of this bacteria has been already obtained n crystalline state by Hagihara in another way. B. Hagihara, *Proc. Jap. Acad.*, **27**, 346 (1951); *Ann. Rep. Scient. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954).

10) A.W. van der Haar, "Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und aldehydsaüren", Verlag von Gebruder Borntrager, Berlin, 1920, s. 120.

11) C. J. Lintner und P. Sollied, *Z. ges. Brau.*, **26**, 103 (1903), see (8).

12) C. H. Beaven and E. R. Holiday, *Advances in Protein Chemistry*, **7**, 370 (1952).

13) O. Folin and V. Ciocalteu, *J. Biol, Chem.*, **73**, 627 (1927).

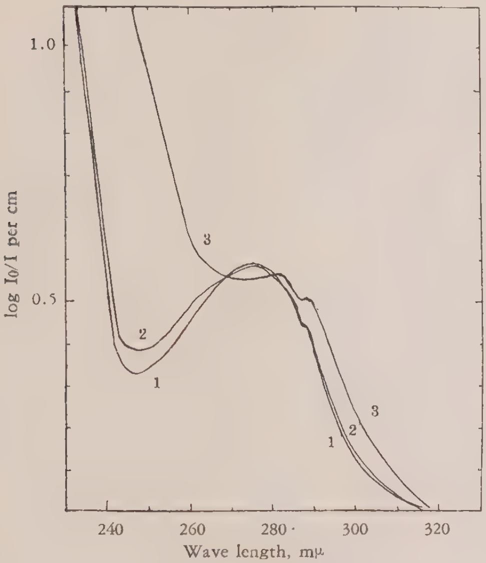

Fig. 1. Ultraviolet absorption of crystalline bacterial α-amylase (0.0231mg of protein N):

(1) in H_2O containing a trace of Ca-acetate, pH 5.8; (2) in M/10 NH^4OH, pH 11.0; amylase, active; (3) in N/10 NaOH, stood for 2 hrs. in order to deactivate completely; Beckman model DU, temp., 14°C.

shown by the equimolar solution of tyrosine and tryptophan under the same condition was 3.2 : 1 (tyrosine : tryptophan).

Stability of the amylase at various pH values is shown in Fig. 2.

2. Effect of Phenol Reagent. The relation between the color development and the change in enzyme activity by the addition of phenol reagent to the amylase solution was investigated. To 1 ml. of amylase solution, 8 ml. of various concentrations of carbonate or bicarbonate solution and 0.5 ml. of phenol reagent were added, and the color value and the amylase activity of the solution were determined after standing at 18°C. For comparison, the color value of standard tyrosine solution was also determined under the same condition. As shown in Table I, when the color value reached to about a third of the full color value obtained for the amylase, which had been previously denatured in sodium hydroxide solution, the amylase was completely inactivated. However, up to this color value the rate of inactivation of the amylase was slower than the rate of the color development. This was, especially, evident in solutions of higher concenration of carbonate. Table I also showed that the color value developed in carbonate solution is only 40% of the full color value, even in a carbonate solution of 0.7 mole concentration.

3. Effect of FDNB. Effect of FDNB on amylase was studied under a mild condition for the reaction. 2 ml. of amylase, 1 ml. of bicarbonate or carbonate solution, and 0.2 ml. of 5% FDNB solution in 96% alcohol were placed in test tubes with stopper and the tubes were shaken at 13°C or at 37°C. The amylase activity change of the mixture was determined at intervals of thirty minutes. As shown in Fig. 3, the amylase inactivation in bicarbonate solution was slight, but remarkable in solutions containing carbonate and the degree of inactivation increased with increase in pH value.

After shaking the mixture of amylase and FDNB in the presence of bicarbonate solution for two or four hours at 37°C, the amylase activity of the supernatant solution of the mixture was investigated in regard to pH-activity curve and pH-stability curve (Fig. 4 and Fig. 5). Addition of FDNB to the enzyme reaction mixture of the control amylase even in concentration of 80 γ per ml., which corresponded about five to ten times the concentration in the enzyme reaction mixture of the FDNB-amylase solution, had no

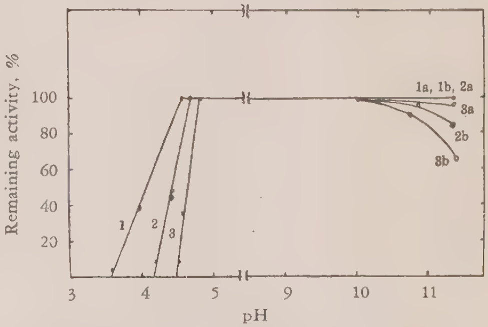

Fig. 2. Stability of crystalline bacterial α-amylase at various pH values.

(amylase 240 LSV/ml. in solutions buffered with N/10 acetic-acetate, M/20 NaH_2PO_4-Na_2HPO_4, M/10 NH_4Cl-NH_4OH, M/10 $NaHCO_3$-Na_2CO_3 and with M/5 NH_4OH (a), M/10 Na_2CO_3-NaOH (b) at higher than pH 11.0) : 1 hr. at 18°C (1), 30 C (2), and at 40°C (3).

Table I

Color Development and Activity Change of Amylase upon Addition of Phenol Reagent
(1 ml. of amylase (1554 LSV), 8 ml. of $NaHCO_3$ or Na_2CO_3 solution,
and 0.5 ml. of phenol reagent, temp., 18°C).

Concentration of	Amylase							
$NaHCO_3$, M/l	0.075	0.025	—	—	—	—	—	—
Na_2CO_3, M/l	0.013	0.038	0.05	0.075	0.10	0.125	0.73	0.73
pH after addition of phenol reagent	7.8	9.6	10.4	10.6	10.8	—	—	—
Ex. per 1.7 cm at 670mμ after 30 mins.	0.10	0.14	0.16	0.19	0.24	0.29	0.35	0.87*
,, 90 ,,	0.20	0.21	0.21	0.22	0.26	0.30	0.36	—
Amylase activity (total), LSV after 30 mins.	1140	1040	956	732	50.6	2.0	0	—
,, 90 ,,	950	856	776	610	20.8	0	—	—

Concentration of	Tyrosine (40 γ)					
$NaHCO_3$, M/l	0.075	0.025	—	—	—	—
Na_2CO_3, M/l	0.013	0.038	0.05	0.075	0.125	0.73
pH after addition of phenol reagent	7.8	9.8	10.6	10.8	—	—
Ex. per 1.7 cm at 670mμ after 30 mins.	0.20	0.32	0.36	0.37	0.38	0.42
,, 90 ,,	0.22	0.33	0.36	0.37	0.38	0.42

* Full color value.

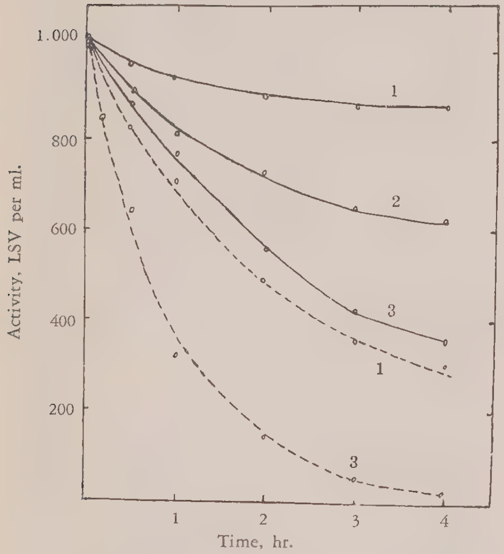

Fig. 3. Effect of FDNB on amylase activity.

2 ml. of amylase (total activity, 3290 LSV), 1 ml. of M/10 $NaHCO_3$ (1), or of pH 8.9 (2) or pH 9.9 (3) of M/10 $NaHCO_3$-M/20 Na_2CO_3 buffer solution, and 0.2 ml. of 5% FDNB in 96% alcohol; at 13°C, --- at 37°C.

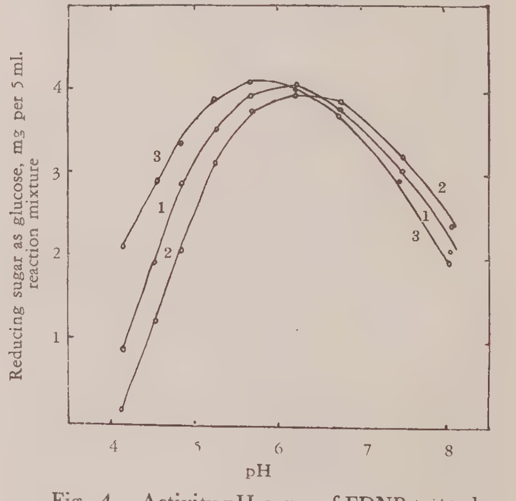

Fig. 4. Activity-pH curve of FDNB-treated amylase.

(1) at 2 hrs., (2) at 4 hrs. of broken line-curve 1 in Fig. 3, and (3) control amylase. Reaction mixture; 5 ml. of 2% soluble starch, 4 ml. of M/15 acetate or M/20 phosphate buffer, and 1 ml. of amylase possessing approximately 10 LSV activity; 40°C, 7.5 mins.

effect on the pH-activity curve. Consequently, it was evident that the decrease in activity and stability in the acid side of pH less than 6 is due to the result of reaction of amylase with

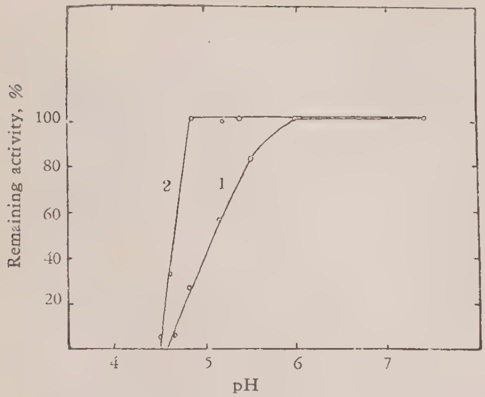

Fig. 5. Stability of FDNB-treated amylase in acid side.

(1) at 4 hrs. of broken line-curve 1 in Fig. 3, and (2) control amylase; 40°C, 1 hr. in the same condition as in Fig. 2.

FDNB.

The supernatant solution (2 ml.) of the mixture of amylase and FDNB in the presence of bicarbonate was acidified to pH 2.0 with hydrochloric acid after shaking for two hours at 37°C. The resulting flocculent precipitate was collected by centrifugation and washed at first with a small amount of distilled water, then with absolute alcohol, and dried under reduced pressure. The dried substance was hydrolized with 1 ml. of 6 N hydrochloric acid in a sealed tube for twenty-four hours at 105°C. The hydrochloric acid was then removed under reducd pressure and the hydrolyzate was analyzed by paperchromatography using butanol as the developer. The chromatogram showed only one yellowish spot corresponding to ε-DNP-lysine.

4. Effect of Iodine. Effect of iodine on amylase protein was investigated. Two milliliter each of amylase solution was placed in test tubes and 3 ml. of buffer solution of various pH values were added. Keeping the test tubes in ice-water, 1ml. of iodine solution (N/200 I_2−M/100 IK) was added to each (2°C). After standing for 15 minutes, 0.1 ml. aliquot of the solution was analyzed for amylase activity, and the remaining solution was titrated with N/200 sodium thiosulfate after addition of 0.1 ml. of 10% sulfuric acid and 0.05 ml. of 1% soluble starch.

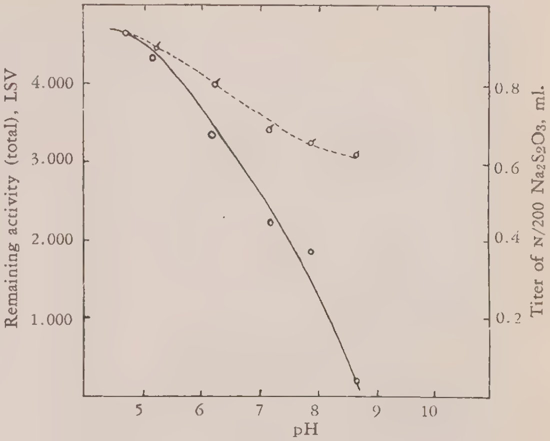

Fig. 6. Iodine consumption and activity change of amylase.

amylase used, 4800 LSV; —— activity, - - - iodine consumption.

In experiments of pH values less han 6, as shown in Fig. 6, iodine consumption was low and the enzyme inactivation was also slight, but both the iodine consumptionand the degree of amylase inactivation increased rapidly with increase in pH value of the solution. However, they were not always in parallel and the degree of inactivation per unit iodine consumption rather increased at the higher pH values. Effect of iodine on amy-

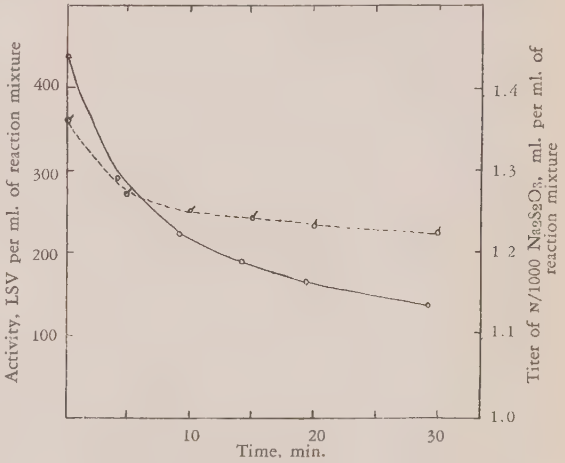

Fig. 7. Iodine consumption and activity change of amylase as a function of time.

5 ml. of amylase (total activity, 7360 LSV), 7 ml. of M/10 phosphate buffer (pH 7.3), and 5 ml. of ca. N/200 I_2-M/100KI; 2°C; · activity, - - - titer of N/1000 $Na_2S_2O_3$.

Table II
Some Properties of Iodinated Amylase

	Active	Alkali-denatured	Active (control)
Amylase (activity, 910 LSV/ml.), ml.	9	9	9
M/20 Na_2CO_3 added, ml.	1.5	1.5	1.5
N/100 I_2–M/50 KI added, ml.	3	3	(H_2O, 3 ml.)
Temp., C	2	12	
Reaction time, min.	5	19	
aliquot taken, ml.	11.5	11.5	11.5
Ml N/100 I_2 removed (titer)	2.17	1.02	
Final volume, ml. after adjusting the pH to 7.6	14.3	11.8	11.7
Amylase activity, LSV/ml.	31	—	600
Color value in tyrosine equivalent, γ/ml.			
Total	28.4	22.4	32.4
TCA soluble portion	5.8	6.1	0
,, after treating with bact. protease (40°C, 60 min.)	5.2	19.2	0
Precipitation with TCA of the protease-treated solution	卌	negative	卌
Adsorption on cornstarch	+	*	+

* When a small amount of ammonium sulfate was added, flocculent ppt. occurred even in a sol. of pH 8.0.

lase in regard to time was also investigated. The result is shown in Fig. 7. From experiments undertaken for Fig. 7 it was found that one atom equivalent of iodine approximately inactivates 1,000 g of crystalline bacterial amylase at pH 7.3.

Some properties of iodine-treated amylase were investigated. In the experiment an aliquot of the iodine-amylase solution was titrated with sodium thiosulfate in the presence of sulfuric acid and soluble starch, and to the remaining solution an equivalent amount of thiosulfate was added at once without addition of sulfuric acid or soluble starch. This latter portion was used for further investigation of its properties. As will be seen in Table II, the iodinated amylase, which had previously been denatured with alkali, was digested by bacterial protease, but the amylase, which had lost most of the amylase activity by treating directly with iodine, was not digested by the protease. Furthermore, this iodine-treated amylase was found to be adsorbed reversibly on cornstarch similar to active amylase. After addition of cornstarch (10%) to the iodine-treated amylase solution (pH 7.6) in the presence of ammonium sulfate (18%), the temperature of the mixture was lowered from 35°C to 8°C, and then conversely raised from 8°C to 35°C. When trichloroacetic acid (TCA) was added to the supernatant solution of the mixture at each temperature, it was found that reasonable precipitation occurs at 35°C, but not at 8°C.

Discussion

In the present study it was found that the inactivation of bacterial α-amylase by phenol reagent, FDNB, and, iodine increases with the increase in pH value although the tendency of inactivation is more or less varied. This fact suggested that the amylase contains chemical reactive groups which are possible to react easily with these reagents at higher pH values. The amylase is not inactivated by sulfhydryl reagents such as *p*-chloromercuri-benzoate.[14] Thus, it may be possible to assume that the reactive group of the amylase in solutions of higher pH value is phenolic group of tyrosoine and the inactivation of amylase by the reagents is mainly due to chemical changes of the tyrosyl group although there may be a share due to chemical change of tryptophanal group. However, the fact that in the inactivation of amylase by phenol reagent, the color development to a certain extent precedes the inactivation of the amylase, seems to signify that a small portion of tyrosyl groups is possible to be changed without much effect on the activity of amylase. The experiment on ionization of tyrosyl groups of the bacterial α-amylase protein has not yet been undertaken, but, at any rate, it may be conceived from Fig. 1 that the phenolic hydroxyl group of tyrosine in amylase protein ionizes to a certein extent in the vicinity of the stability limit of the amylase in alkaline sides.

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[14] Unpublished.

Inactivation of amylase by iodine may be due to iodination[15] of tyrosine. However, in this case iodination of tryptophan is not to be overlooked although the extent of contribution to the inactivation of amylase is not clear from the present study. The iodinated amylase, treated to a state in which the amylase had lost most of the activity, was not digested by bacterial protease. This fact forms a contrast to the fact that calcium-removing agents, such as citrate, oxalate, or ethylene diamine tetraacetate, alter the amylase protein into bacterial protease-digestible form as soon as they inactivate the amylase.[3] Generally, protein changes into a easily protease-digestible form by denaturation, which is attributed to the rupture of secondary intramolecular bonds of the protein. In the study of denaturation with various enzymes Okunuki and his coworkers[16] demonstrated that denatured enzyme protein can be detected by the susceptibility to bacterial protease (*Bac. subtilis*), which easily hydrolyzes the denatured protein into trichloroacetic acid-soluble form. Nevertheless, it is certain that during the inactivation by iodine under a certain condition the molecular structure of the amlyase protein at least does not change to an extent as to be bacterial protease-digestible.

Chemical changes of tyrosyl or tryptophanal groups of protein may be possible without much effect on the structure of the protein. This is conceivable from the present study that after inactivation by iodine under a certain condition it was still possible for the amylase protein to be adsorbed on cornstarch reversibly similar to an active amylase, and also from the facts that Maekawa[17] obtained a crystalline bacterial amylase, which had lost most of its activity by oxidation with periodate under a certain condition, and that partially iodinated human serum albumin[18] and partially oxidized chymotrypsin[19] have been obtained in crystalline form.

The reaction of amylase with FDNB may be classified at least into two types: a reaction (1) which occurs in a comparatively low pH solution introducing a change in the stability of the amylase but not in the activity itself, and another (2) which tends to take place at a comparatively high pH value causing inactivation of the amylase. In the supernatant solution of reaction mixture of amylase with FDNB in the presence of bicarbonate, in which activity of the amylase had still remained but its stability had been altered, only one DNP-derivative corresponding to ε-DNP-lysine was found clearly after hydrolysis. Thus, it may be possible to assume that the reaction (1) is concerned with some portion of ε-amino groups of lysine in the amylase protein and the reaction (2) with phenolic hydroxyl group of tyrosine forming O-DNP-tyrosine, or with α-amino group of terminal amino acid, arginyl, or histidyl groups of the amylase, although a decisive conclusion must be left to further study.

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[15] Iodine is possible to react with histidyl group beside tryptophanal and tyrosyl groups. K. J. Bunings, *J. Am. Chem. Soc.*, **69**, 205 (1947).

[16] K. Okunuki, B. Hagihara et al., *Symposia on Enzyme Chem. (Japan)*, **9**, 1 (1954); *Ibid*, **10**, 143 (1954).

[17] K. Maekawa and K. Kitazawa, *Proc. Jap. Academy*, **29**, 353 (1953).

[18] W. L. Hughes, Jr., and R. Streessle, *J. Am. Chem. Soc.*, **72**, 452 (1952).

[19] E. F., Jansen, A. L. Curl, and A. K. Balls, *J. Boil Chem.*, **189**, 671 (1951).

Summary

1. The three reagents (Folin's phenol reagent, fluorodinitrobenzene, and iodine) inactivate bacterial α-amylase slightly in an acid, but, remarkably in an alkaline solution.

2. Phenol reagent attacks the amylase protein with developing color, followed by a gradual loss of enzymic activity.

3. Iodine is consumed by the amylase increasing at higher pH values of solution and inactivates the amylase at greater rate than that of the reagent consumption.

4. Fluorodinitrobenzene seems to react in dual ways, one, changing pH-stability curve of the amylase without decreasing the enzymic activity and the other, inactivating the amylase activity itself.

Acknowledgements

The auther wishes to thank Prof. Dr. I. Yamasaki of Kyushu University and Prof. Dr. J. Fukumoto of Osaka City University, for their invaluable advice throughout this work.

Biochemical Studies on Vitamin B_{12}. Part X

On Vitamin B_{12}-like Activity of Cobalt-Porphyrin-Derivative

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The alkali- and thermo-stable substance indicating vitamin B_{12}-activity in plant kingdom was confirmed to be a porphyrin-derivative containing cobalt by paper partition chromatography and bioautography. Next, Culture experiments on *Euglena* and *Lactobacillus leichmannii* with Cobalt-porphyrin-derivative prepared from radish leaves were always successful by vitamin B_{12}-free basal medium when supplemented with 1,2-diamino-4,5-dimethylbenzene or 5,6-dimethylbenzimidazole.

From these results, thc physiological role of Co-porphyrin-derivative as one of the active components effecting on the intermediary metabolism of vitamin B_{12} seems to be established.

Introduction

Since the year 1950, the authors have reported several studies on the biosynthesis *de novo* of vitamin B_{12} and effect of the vitamin on energy metabolism. In the mean while, many papers appeared indicating that there may be alkali- and thermo-stable compound similar to vitamin B_{12} in plant kingdom. Recently, the present authors attempted the physiological studies on the intermediary metabolism of vitamin B_{12} formation.

At first, vitamin B_{12} activity of Japanese radish-leaves was tested, and an alkali-stable compound was detected from radish-leaf preparation by bioautography.[1] On the other hand, chromatographic comparison of radish-leaf preparation and chlorophyll was repeated, and it was found that the above compound may be a kind of Cobalt-porphyrincompounds.[2, 3]

In 1950,[4] the present author reported biosynthesis of vitamin B_{12}-active substances from 5, 6-dimethylbenzimidazole by *Streptomyces.* In 1951,[5] Woolley stated that the only active compound in replacing vitamin B_{12} for *Euglena* may be 1, 2-diamino-4, 5-dimethylbenzene, but the replacement was incomplete because of the low solubility of the substance and the minute equivalent amount of vitamin B_{12} which it replaces.

Therefore, experiments on *Euglena* and *Lactobacillus leichmannii* with Co-porphyrin-derivative were repeated by vitamin B_{12}-free basal medium supplemented with 1, 2-diamino-4, 5-dimethylbenzene or 5,6-dimethyl-benzimidazole,[6] and complete growth response of the microbes was observed.

From these findings, it seems to be proved that Co-porphyrin-derivative may be one of the active components effecting on the intermediary metabolism of vitamin B_{12} in these organisms.

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* Biochemical Laboratory, Tokyo University of Agriculture.

[1] Y. Sahashi and T. Muto, Japan Vitamin Soc., Tokyo Meeting Sept. 18th, 1954.

[2] Y. Sahashi and T. Muto, *Proc. Vitamin B Res, Comm., (Japan)* **73**, 6 (1954).

[3] Y. Sahashi and T. Muto, *ibid*, **74**, 11 (1954).

[4] Y. Sahashi, M. Mikata and H. Sakai, *Bull. Chem. Soc. (Japan)*, **23**, 247 (1950).

[5] D.W. Woolley, *J. Exptl. Med.*, **93**, 13 (1951).

[6] Y. Sahashi and T. Muto, *Proc. Vitamin B Res. Comm.*, **76**, 1 (1955).

Experimental

(1) The Test of Vitamin B_{12}-activity in Radish-leaves

At first, 100g of radish-leaves were homogenized with 300cc of distilled water. To the homogenate thus obtained a few cc of toluene was added and the mixture was treated with 5g of takadiastase at 37 °C. After 48 hours, the residual substances in the decomposition products were removed by centrifuge and 320cc of clear filtrate (radish-leaf preparation) was obtained. To 100cc of radish-leaf preparation 800cc of 1/5N NaOH was added and after heating at 100 °C for 30 min. the solution was neutralized to pH 6.0. The radish-leaf specimen thus obtained indicated more remarkable increase in the growth of *Euglena* than the ordinary untreated one.[7] On the other hand, further heating experiments with autoclave (20 lbs., 10 min.) before takadiastase treatment were repeatedly carried out. The variation in the growth of *Euglena* was accurately measured by the optical density in the electrophotometer and the vitamin B_{12}-activity was estimated on the eluate of spot-area of R_F 0.65–0.75 of paper chromatograms. The repeated experiments under different conditions as mentioned above showed always further additional effect on the growth of *Euglena*. (Table I)

Table I
The Deviation of the Optical Density of Vitamin B_{12}-Activity after various Treatments under different Conditions.

R_F in the Experiments I, II		Readings of Optical Density			
		Before Treatment	After Alkali-Treatment	After Autoclave-Treatment	After Treatment by Autoclave and then with Alkali
I	0.65	0.245	0.365	—	—
	0.75	0.245	0.452	—	—
II	0.65	0.245	—	0.335	0.585
	0.75	0.245	—	0.360	0.545

(2) Bioautography of Radish-leaf Preparation

Toyo No. 2 filter paper moistened with 0.66M KH_2PO_4 buffer (pH 4.0) was cut to measure 2 × 30 cm and 0.1 cc/spot was applied to the paper at the spot marking the original. Standard vitamin B_{12} (0.01 mγ/spot) was also placed in the ordinary way. The chromatographic experiments of radish-leaf preparation was developed in the *n*-butanol-acetic acid-water (4:1:2) system as mobile phase by ascending method, and the vitamin B_{12}-activity was tested after drying at 90–100 °C for 5min. It was shown that materials contained alkali-stable and thermo-stable substance on the paper strip (R_F 0.65–0.75) similar to the activity of vitamin B_{12} (Figs. 1–2).

(3) Determination of Cobalt in Vitamin B_{12}-active Region of Radish-leaves

To 50g homogenized radish-leaves 40cc conc. nitric acid (Sp. Gr. 1.39) was added and after evaporating the decomposition product was treated with 30 cc of hydrogenperoxide and 5 cc of hydrochloric acid. After 6–12 hrs. standing, the residual product was removed by centrifuge, and the filtrate was evaporated to dryness. The syrupy material thus obtained was dissolved in 1cc water and the appearance of Cobalt was tested by narrow strip paper chromatography with the mobile phase composed of *n*-butanol-12N HCl (80:20) After drying at 30–40 °C for 12hrs. Cobalt ion in the paper strip was tested by 0.2% nitroso R salt spray. After drying at 90–100 °C for 10min. the Cobalt ion region was found to be located in the paper strip (R_F 0.05–0.25), and the dye was eluted with 1M H_2SO_4 for 3 hrs from the paper. As described above, Cobalt in radish-leaves was determined from the optical

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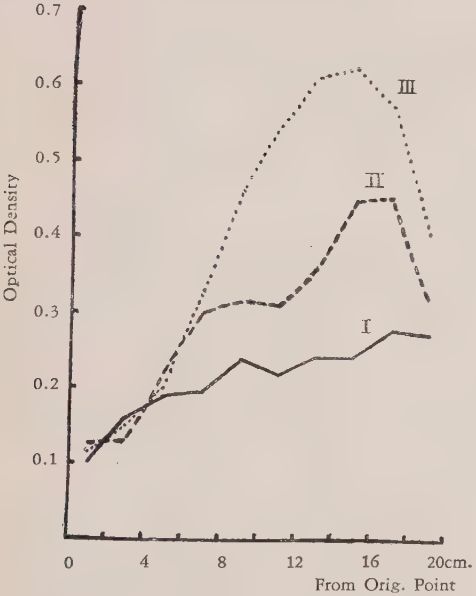
[7] K. Ueda, Recent Quantitative Method of Vitamins (Ishiyaku Press Co.) p. 95 (1954); B_{12} and APF (Nankodo Co.) p. 41 (1955).

Table II
The Content of Cobalt in Radish-leaf preparation.

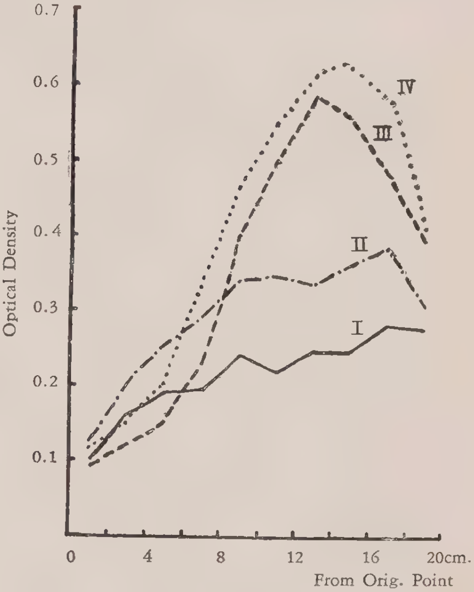
Sample	Cobalt γ/100g, dry matter	Vitamin B_{12} γ/100g, dry matter	Vitamin B_{12} γ/100g, fresh leaves
Radish leaves	3–6	—	0.02
Radish-leaf preparation before treatment	4.0	3.11	—
After alkali treatment	3.0	1.33	—
After autoclave treatment	—	0.6–2.06	—
After autoclave and alkali treatment	3–5	0.04	—



I: Before Treatment.
II: After Alkali-Treatment
III: Vitamin B_{12} (0.01mγ/spot).

Fig. 1. Bioautogram of Radish-leaf Preparation after Alkali-treatment.

density of the dye at 430mμ as shown in Table II.[8–13]



I: Before Treatment.
II: After Autoclave-Treatment.
III: After Treatment by Autoclave and then with Alkali.
IV: Vitamin B_{12} (0.01mγ/spot).

Fig. 2. Bioautogram of radish-leaf preparation after Autoclave-treatment.

(4) Comparison of Radish-leaf Preparation and Chlorophyll

From the repeated experiments, it was found that the radish-leaves preparation may contain porphyrin compounds. Then, chlorphyll a, b preparations were freshly prepared from radish-leaves by the ordinary process, and both specimens were accurately tested by spectrography

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[8] J.A. Dean, *Analyt. Chem.*, **23**, 1096 (1951).
[9] H. Almond, *ibid.*, **25**, 166 (1953).
[10] K.J. McNaught, *New Zealand J. Sci. Technol.*, **30A**, 109 (1949).
[11] J. Tamura, *Analyt. Chem. Japan*, **1**, 117 (1952).
[12] Y. Oka and M. Miyamoto, *ibid.*, **2**, 316 (1953).
[13] T. Muto and Y. Sahashi, *Agr. Chem. Soc. Japan*, Gen. Meeting 4th, April (1954).

Table III

Comparison of Spectrograms and Chromatograms.

	Radish-leaf-Preparation	Chlorophyll-Preparation	Literature[15]
Absorption Max.	406mμ (10mg%EtOH)	410mμ (10mg%EtOH)	—
β-Carotene	R_F 0.96	R_F 0.96	R_F 0.98
Xanthophyll	R_F 0.68	R_F 0.68	R_F 0.69
Chlorophyll a	R_F 0.38	R_F 0.38	R_F 0.42
Chlorophyll b	R_F 0.16	R_F 0.16	R_F 0.21

and by ascending chromatography in the petroleum ether-ether (3:1) system as mobile phase. (Table III)[14–15] From the above findings, it seems to be clear that the radish-leaf preparation may be similar to chlorophyll complexes.

(5) Co-Porphyrin-Derivative

To 10 g radish-leaf-powder prepared by air dryer at 70°C, 30cc of 80% acetone was added and macerated for several hours. The insoluble substances were removed by filtration by the pump and the almost white residues were repeatedly washed with 45cc of the same solvent. The yellowish green acetone solution was evaporated at 60–70°C under diminished pressure to dryness, yielding a syrupy material. The yellowish green compound was treated with 10mg $CoCl_2$ in 100cc methanol at 70°C. After 1 hr. heating, methanol was removed, and the resulting bluish green mass was subsequently purified repeatedly by washing with water and by extracting with ether.

Then, after adjusting to pH 8.0 with alcoholic NaOH, the solution was added with water and the water fraction separating from ether layer was washed with few cc of petro-ether: ether (3:1) repeatedly, and the water solution of Co-porphyrin-derivative (Na salt) was obtained.

Absorption spectra:

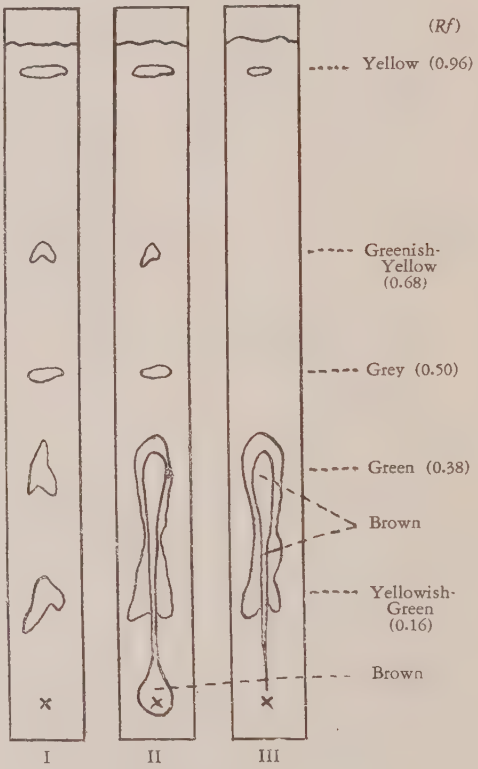
406, 510, 655mμ (10mg % EtOH)
410mμ (10mg % EtOH) ...... Chlorophyll-preparation
510mμ (0.1 M H_2O) .... $CoCl_2 . 7H_2O$

Analysis of Co:

Sample	Co in Co-Porphyrin-Derivative	Inorg.-Co
100mg	4.85γ (0.0049%)	0

Chromatographic experiments of these compounds are shown in Fig. 3.

From the above results the Co-porphyrin-prepa-



I: Chlorophyll-Preparation.
II: Co-Porphyrin-Preparation (before washing with water).
III: 〃 (after washing with water).
Mobile Phase: Pet. ether-Ether (3:1).

Fig. 3. Chromatograms of Co-porphyrin Preparation and Chlorophyll Preparation.

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14) L. Bauer, *Naturwiss.*, **39**, 88 (1952).
15) S. Hattori, Plant Pigments (Iwanami Co.) p. 489 (1936).

ration may be Cobalt conjugate of porphyrin series.

(6) Bioassay of Co-Porphyrin-Derivative

Euglena: Experiments were carried out by the ordinary method with Co-free basal medium composed of:

	(in 1*l*)
$(NH_4)H_2PO_4$	0.8 g
K-citrate monohydrate	0.3 〃
$MgSO_4 \cdot 7H_2O$	0.2 〃
Na-butyrate	2.0 〃
Na-glutamate	1.0 〃
$CaCl_2$	0.1 〃
$FeSO_4 \cdot 7H_2O$	20 mg
$MnSO_4 \cdot H_2O$	6 〃
$ZnCl_2$	0.8 〃
$Na_2MoO_4 \cdot 2H_2O$	1 〃
$CuSO_4 \cdot 5H_2O$	0.08 〃
Thiamin chloride	0.1 〃
	(pH 6.5)

The results are shown in Tables IV–V.

In the second experiment with *Euglena*, further observation was repeated in the vitamin B_{12} containing medium supplemented with Cobalt-

Table IV

Effect of Co-Porphyrin-Derivative on Optical Density of the Growth Promotion in *Euglena*.

	Optical Density	
	After 96 hrs.	After 120 hrs.
none	0.205	0.275
Co-porphyrin (7 mg/l)	0.215	0.310
B_{12} (0.1γ/l)	0.630	0.680
B_{12} (〃)+$CoSO_4$(5 mg/l)	—	0.740
B_{12} (〃)+Co-porphyrin (7 mg/l)	0.850	0.895
B_{12} (〃)+Co-porphyrin (21 mg/l)	0.875	0.980

Table V

Effect of Co-Porphyrin Derivative upon the Growth of *Euglena* with vitamin B_{12} containing Medium.

	Deviation of Optical Density by various Amount of Vitamin B_{12}			
	0.02 mγ/cc	0.05 mγ/cc	0.07 mγ/cc	0.1 mγ/cc
$CoSO_4$ 5mg/l	0.375	0.425	0.433	0.437
Co-porphyrin 7 mg/l	0.400	0.520	0.550	0.566
Co-porphyrin 21 mg/l	0.440	0.605	0.635	0.650

Table VI

Effect on $CoSO_4$-Chlorophyll Supplement on the Growth of *Euglena* in the Vitamin B_{12} containing medium.

	Optical Density		
	After 72 hrs.	After 96 hrs.	After 120 hrs.
Chlorophyll (7 mg/l)	0.130	0.195	0.240
Chlorophy (7 mg/l)+$CoSO_4$ (5 mg/l)	0.116	0.175	0.240
B_{12} (0.1γ/l)	0.265	0.600	0.680
B_{12} (0.1γ/l)+Chlorophyll (7 mg/l)	0.215	0.440	0.735
B_{12} (0.1γ/l)+$CoSO_4$ (5 mg/l)	0.245	0.520	0.735
B_{12} (0.1γ/l)+$CoSO_4$+(5 mg/l) Chlorophyll (7 mg/l)	0.275	0.720	0.875

Table VII

Effect of Co-Porphyrin-Derivative upon the Growth of *Chlorella*.

Cobalt Compound supplemented	Amount of Cobalt compound added		Variation of the Growth in *Chlorella*.	
	0 mg/100 cc		100	
$CoCl_2$	10 〃		102	
	100 〃		83	
	1000 〃		43	
	0 〃		100	
$CoSO_4$	0.07 〃		111	
	0.7 〃		101	
	7.0 〃		100	
	Co-Porphyrin	B_{12}	Exp. I	Exp. II
	0.0 mg/100cc.	0.0 γ/100cc.	100	100
Co-porphyrin + Vitamin B_{12}	0.0 〃	0.01 〃	119	106
	0.0 〃	0.5 〃	124	124
	0.7 〃	0.0 〃	97	86
	2.1 〃	0.0 〃	99	91
	0.7 〃	0.01 〃	140	117
	2.1 〃	0.01 〃	142	125

Table VIII

Vitamin B_{12}-like Activity of Co-Porphyrin-Derivative supplemented with 5,6-Dimethylbenzimidazole (D.B.I.) on *Euglena*.

	Optical Density			
	After 48 hrs	After 72 hrs	After 96 hrs	After 120 hrs
none	0.105	0.155	0.205	0.275
D.B.I. (3mg/l)+$CoSO_4$(5mg/l)	0.130	0.180	0.233	0.297
D.B.I. (3 mg/l)+Co-Porphyrin (21 mg/l)	0.175	0.335	0.475	0.577
B_{12} (0.1γ/l)+$CoSO_4$(5mg/l)	0.265	0.515	0.597	0.632

Table IX

Optical Density of the Growth Response in *Euglena* with Co-Porphyrin-Derivative containing 1,2-Diamino-4,5-dimethyl benzene. (D.D.B.)

	Optical Density			
	After 48 hrs	After 72 hrs	After 96 hrs	After 120 hrs
none	0.105	0.155	0.205	0.275
D.D.B. (3mg/l)+$CoSO_4$(5mg/l)	0.125	0.175	0.223	0.285
D.D.B. (3mg/l)+Co-Porphyrin (21mg/l)	0.173	0.325	0.467	0.566
B_{12} (0.1γ/l)+$CoSO_4$(5mg/l)	0.265	0.515	0.597	0.632

sulfate and chlorophyll. The growth promotion in *Euglena* indicated also more increased response than the ordinary vitamin B_{12} containing medium. (Table VI)

Chlorella: In the third experiment, further repeated studies were made on the growth of *Chlorella* in the Knop's revised medium composed of 500 mg KNO_3, 125 mg KH_2PO_4, 250 mg $MgSO_4 \cdot 7H_2O$, and 3mg $FeSO_4 \cdot 7H_2O$ in 100cc (pH 5-6).[16] After 7-14 days culture at 23-28°C in CO_2 atmosphere under irradiation, the growth of *Chlorella* was measured by the reading of optical density in electophotometer. (Table VII)

(7) Role of Co-Porphyrin-Derivative in the Intermediary Metabolism of Vitamin B_{12}

Based on the above various researches, further

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[16] J.S. Burlew, Algae Culture (Washington Press), p. 38 (1953).

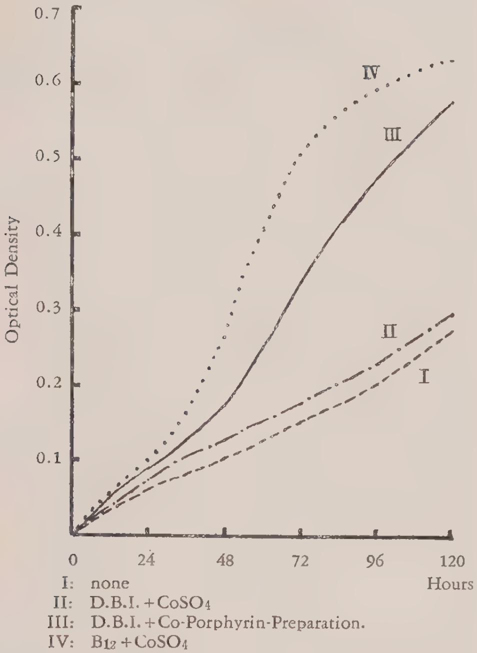

Fig. 4. Variation of Optical Density effected by Co-Porphyrin-Derivative with 5,6-Dimethylbenzimidazole in the Growth of *Euglena*.

experiments with Co-porphyrin-derivative in the intermediary metabolism were recently carried out by completely vitamin B_{12}-free basal medium mentioned above. At first, 5,6-dimethylbenzimidazole was supplemented to Co-porphyrin-preparation, and vitamin B_{12}-like activity was positively confirmed in the response of *Euglena*. (Table VIII, Fig. 4)

On the other hand, experiments on *Euglena* with Co-porphyrin-derivative were again carried out by Vitamin B_{12}-free basal medium supplemented with 1,2-diamino-4,5-dimethyl benzene, and the growth response of the microbe was also recognized (Table IX)

Moreover, similar experiments with *Lactobacillus leichmannii* were also repeated at 37°C, for 40-48hrs. by completely vitamin B_{12}-free medium[17-19] composed of:

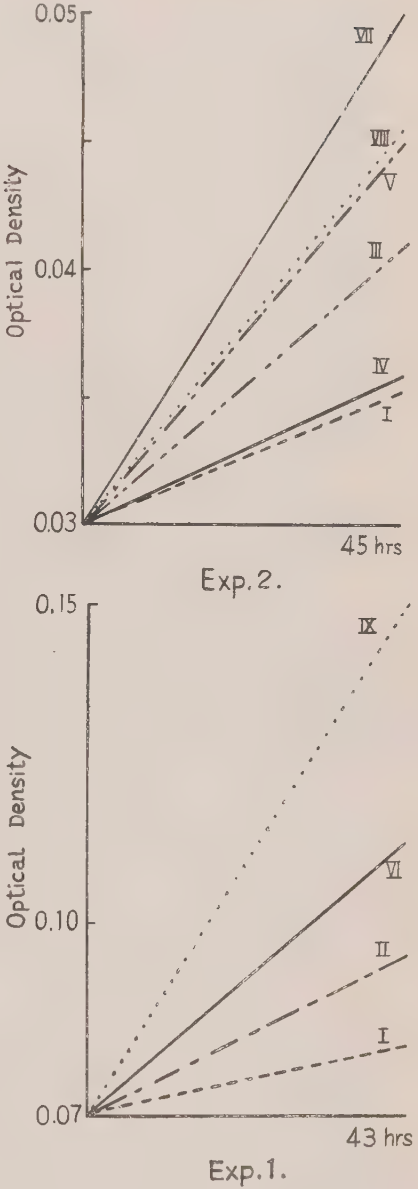

Fig. 5. Variation of Optical Density effectedby Growth-Promotion of *L. leichmannii* supplemented with 5,6-Dimethylbenzimidazole and Co-Porphyrin-Derivative.

Sapplements: Curve I. None; II. D.B.I. (6 mg/l); III. D.B.I. (18 mg/l); IV. Co-Porphyrin (21 mg/l); V. D.B.I. (6 mg/l) + Co-Porphyrin (21 mg/l); VI. D.B.I. (6 mg/l) + Co-Porphyrin (42 mg/l); VII. D.B.I. (18 mg/l) + Co-Porphyrin (126 mg/l); VIII. Vitamin B_{12} (0.02γ/l); IX. Vitamin B_{12} (0.2γ/l)

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17) H. Sakai, *J. Agr. Chem. Soc. Japan* **27**, 377 (1953).

18) T. Matsuda, *Vitamins* **6**, 49 (1953).

19) T. Uwakubo, 3rk. Bioassay Meeting (Tokyo) 5th, Feb. (1955).

Glucose	4 g/1 liter
Casein hydrolysate	1 〃
L-Cystine	20mg/1 liter
L-Tryptophan	20 〃
L-Asparagine	20 〃
Adenine	2 〃
Guanine	2 〃
Uracil	2 〃
Thiamin	200γ/1 liter
Riboflavin	200 〃
Nicotinic acid	200 〃
Ca-pantothenate	200 〃
Pyridoxine hydrochloride	400 〃
Folic acid	20 〃
Biotin	1 〃
p-Aminobenzoic acid	200 〃
Ascorbic acid	100mg/1 liter
Tween 80	0.2 cc
Solution A	1 cc
〃 B	1 cc
	(pH 6.4–6.6)

Solution A: 20 g KH_2PO_4 in 100 cc water.
Solution B: 0.2 g NaCl, 0.3 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.8 g $MnSO_4 \cdot 4H_2O$, 4.0 g $MgSO_4 \cdot 7H_2O$ and 2cc 1N HCl in 100 cc water.

The growth response of *Lact. leichmannii* with 5,6-dimethyl-benzimidazole was positively proved when supplemented with our Co-porphyrin-derivative, indicating vitamin B_{12}-like activity as shown in Fig. 5.

Summary

(1) The alkali- and thermo-stable substance indicating vitamin B_{12}-activity in plant kingdom was confirmed to be a porphyrin-derivative containing Cobalt by paper partition chromatography and bioautography.

(2) Culture experiments on *Euglena* and *Lactobacillus leichmannii* with Co-porphyrin-derivative prepared from radish-leaves were always successful by vitamin B_{12}-free basal medium when supplemented with 1,2-diamino-4,5-dime-thyl-benzene or 5,6-dimethyl-benzimidazole.

(3) From these results, the physiological role of Co-porphyrin-derivative as one of the active components effecting on the intermediary motabolism of vitamin B_{12} seems to be established.

Acknowledgements

The expense of the present investigation was partly defrayed from the Grant in Aid for Fundamental Scientific Research of Education Ministry to which the authors' thanks are due. Their thanks are also due to Messers T. Suzuki, T. Miki, S. Asai, K. Saito, T. Matsushima and Miss M. Nishi for their kindness throughout this work.

Studies on the Essential Oils of Tobacco Leaves

Part I. Acid Fraction

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Japan Monopoly Corporation

Received April 28, 1955

We are studying exhaustively the steam distillable components "Essential Oils" of the formented Virginia tobacco leaves which consist of acids, bases, phenols, carbonyls and neutral compounds. At first we have tried the estimation of volatile organic acids contained in essential oils, and isolated and identified formic, acetic, isobutyric, *trans*-crotonic, *n*-caproic, benzoic and phenylacetic acids by procedures using chromatography and infrared spectra. On the other hand, we also isolated a kind of fatty acid by the same procedure and estimated that it would be a pentoic acid. We could find no report which has proved the presence of *trans*-crotonic, phenylacetic and a pentoic acids in tobacco leaves.

Introduction

The steam distillable components, "Essential Oils" of fermented tobacco leaves are responsible to the specific aroma of smoking tobacco together with resins, waxes and phenolic substances and have hitherto been the object of study by many authors[1),2),3)].

These oils are secreted mainly by the gland hair and are located on the surface of the leaves[4)]. Total amounts of volatile oils are relatively small, varying from about 0.05 to 0.5% of the dry weight of the leaves[4)]. Bacause of their complex composition our knowledge of these leaf components is much limited. In addition, special difficulty is presented by their unstable property, which manifests itself in their tendency to autoxidize, condense and polymerize during the chemical operation for their separation[5)]. According to Schmuck, Kurilo et al., the curing causes further esterification and saturation of double bonds in the oils by estimation of the change of acid value, ester value and iodine value[4)]. Schmuck studied the essential oils of Orient tobacco leaves obtained by the steam distillation and brought about many results. But we have few reports on the essential oils of the fermented Virginia tobacco leaves which are principal material of cigarette manufactured in Japan.

We prepared the essential oils of the fermented Virginia tobacco leaves by steam distillation and separated them into basic, acidic, phenolic, carbonyl and neutral fractions by functional group and tried the identification of chemical constituents contained in each fractions. At first we estimated organic acids of tobacco essential oils. Among the organic acids contained in tobacco essential oils, the

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1) R. F. Dawson, *Ind. Eng. Chem.*, **44**, 267 (1952).

2) H. Brückner, "Biochemie des Tabaks," Verlagsbuchhandlung Paul Parey, Berlin, 1936, p. 207, p. 217.

3) M. Nio, "Tobacco Industry," Sangyô-hyôron-sha, Japan, 1950 p. 410.

4) W.G. Frankenburg, "Advances in Enzymology," **6**, 331 (1946).

5) W.G. Frankenburg, "Advances in Enzymology," **10**. 370 (1950).

Table I

Preparation and Fractionation of Essential Oils

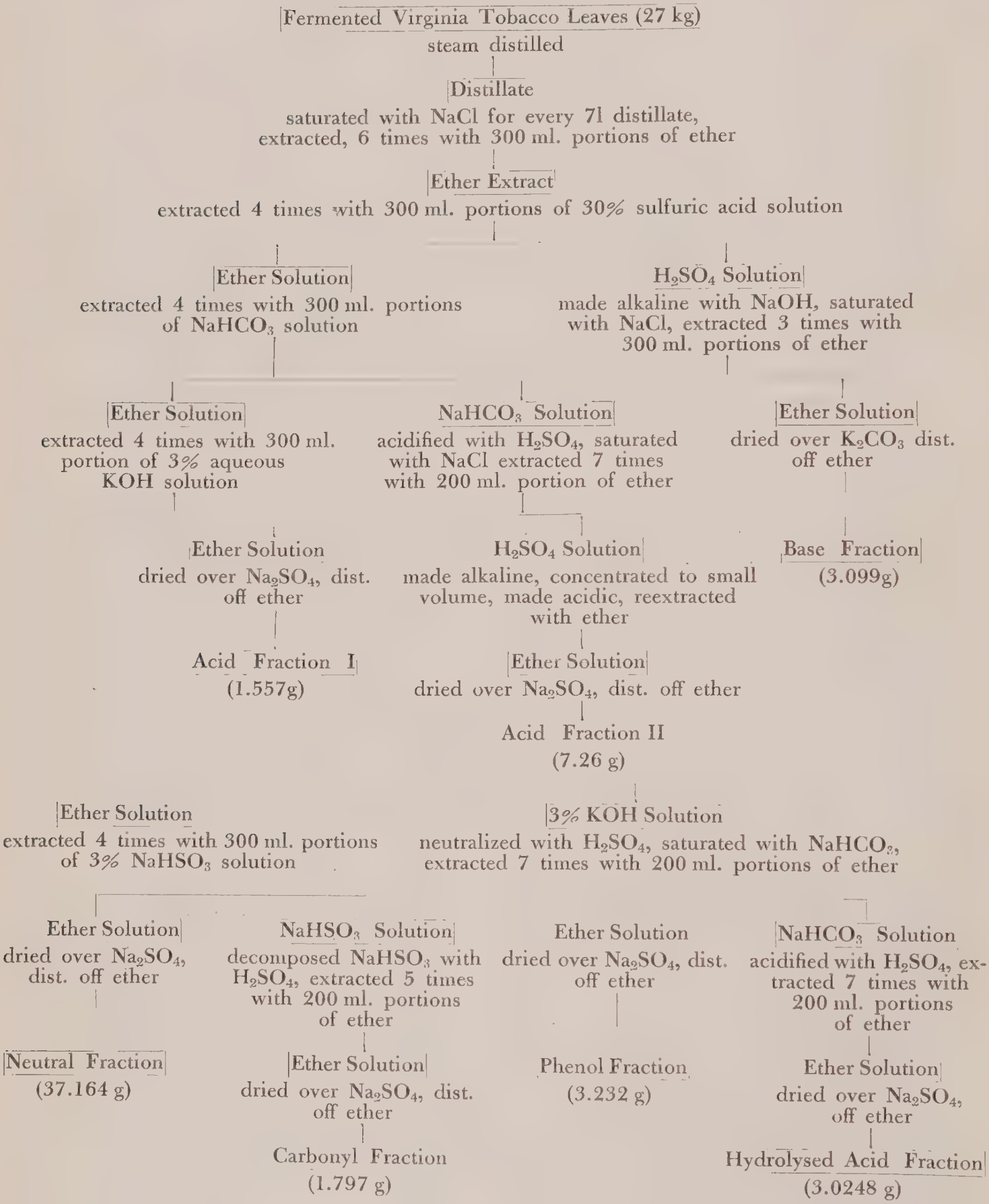
Fermented Virginia Tobacco Leaves (27 kg)
steam distilled
Distillate
saturated with NaCl for every 7l distillate, extracted, 6 times with 300 ml. portions of ether
Ether Extract
extracted 4 times with 300 ml. portions of 30% sulfuric acid solution
Ether Solution
extracted 4 times with 300 ml. portions of $NaHCO_3$ solution
H_2SO_4 Solution
made alkaline with NaOH, saturated with NaCl, extracted 3 times with 300 ml. portions of ether
Ether Solution
extracted 4 times with 300 ml. portion of 3% aqueous KOH solution
$NaHCO_3$ Solution
acidified with H_2SO_4, saturated with NaCl extracted 7 times with 200 ml. portion of ether
Ether Solution
dried over K_2CO_3 dist. off ether
Ether Solution
dried over Na_2SO_4, dist. off ether
H_2SO_4 Solution
made alkaline, concentrated to small volume, made acidic, reextracted with ether
Base Fraction
(3.099g)
Acid Fraction I
(1.557g)
Ether Solution
dried over Na_2SO_4, dist. off ether
Acid Fraction II
(7.26 g)
Ether Solution
extracted 4 times with 300 ml. portions of 3% $NaHSO_3$ solution
3% KOH Solution
neutralized with H_2SO_4, saturated with $NaHCO_3$, extracted 7 times with 200 ml. portions of ether
Ether Solution
dried over Na_2SO_4, dist. off ether
$NaHSO_3$ Solution
decomposed $NaHSO_3$ with H_2SO_4, extracted 5 times with 200 ml. portions of ether
Ether Solution
dried over Na_2SO_4, dist. off ether
$NaHCO_3$ Solution
acidified with H_2SO_4, extracted 7 times with 200 ml. portions of ether
Neutral Fraction
(37.164 g)
Ether Solution
dried over Na_2SO_4, dist. off ether
Phenol Fraction
(3.232 g)
Ether Solution
dried over Na_2SO_4, off ether
Carbonyl Fraction
(1.797 g)
Hydrolysed Acid Fraction
(3.0248 g)

Table II

The Yield and Physical Constants of Tobacco Leaves Essential Oil Fractions

Fraction	Yield (g) for 27 kg tobacco leaves	Percentage for total essential oils	n_D^{20}	d^{20}	Characters of Odor
Acid	9.818	16.89			Sweet, heavy, slightly stimulous aromatic odor, caused by valeric and acetic acid.
Base	3.099	5.33	1.4107	0.7490	Fishy odor, very closely resemble to the odor of Blending Dep. of Cigarette Manufacturing Factory.
Phenol	3.232	5.56		0.8814	Clearly phenolic odor.
Hyd. Acid	3.025	5.20		0.9123	Resemble to Acid Fr. odor, but slightly faint.
Neutral	37.164	63.93			Light aromatic odor, caused by lower esters and alcohols.
Carbonyl	1.798	3.03			Somewhat fishy strong odor, caused by *n*- or isobutyraldehyde.
Total	58.136	100.00			

following have been reported: formic[6], acetic[7], isobutyric[4], isovaleric[3),9], β-methyl-valeric[10], isobutylacetic[11], caprylic[12], palmitic[13] and myristic[14] acids. We have separated organic acids by column chromatography using silicic acid and derived them into *p*-bromphenacyl esters by the usual method[15]. We isolated and identified formic, acetic, isobutyric, *trans*-crotonic, *n*-caproic, benzoic and phenylacetic acids, and else isolated a pentoic acid but could not determine its structure. We did not isolate palmitic and myristic acids from acid fraction, but obtained them from phenol fraction[16]. This is considered to be caused by those palmitic and myristic acids which are weak acids and less soluble in aqueous sodium bicarbonate solution. Many authors reported the presence of formic and acetic acids in essential oils and this agreed with our result. It is interesting that we did not isolate *n*- and isovaleric acids which were reported by many authors. We have no report which has indicated the presence of *trans*-crotonic, *n*-caproic, benzoic and phenylacetic acids in tobacco essential oils.

Experimental

Preparation of Essential Oils:

Flue-cured Virginia Yellow type tobacco leaves, harvested in Okayama district in 1951 and 1952 (smoking leaf, special grade) 250 kg were used. Five to seven kilograms of cuttered tobacco leaves were placed in a distillation-kettle fitted with a water-cooled condenser, and steam distilled for 6–8 hours to get 60–80 l distillate. The distillate was saturated with sodium chloride and extracted with ether. The commercial ether was purified by distillation using packed column ((150 × 3.0 cm diam., stainless ring packing (2.7 mm diam.), reflux ratio 1 : 3, number of theoretical plate ca. 20)). The ether extract was dried over anhydrous sodium sulfate. Removal of ether left the essential oils. To remove ether we used packed column ((100 × 1.2 cm diam., stainless ring packing (2.0 mm diam.), reflux ratio 1 : 3, number of theoretical plate ca. 30)) to prevent the loss of volatile matters. Tobacco essential oils had sweet aroma similar to raisins (n_D^{20}, 1.4131 : d^{20}, 0.829 : yield 0.215%).

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[6] A. Schmuck and S. Kahirin, *U.S.S.R. State in et Tobacco Inv., Bull.*, **69**, 29-32 (1930); *C. A.*, 3124 (1931).

[7] K. Yamafuji, *J. Agr. Chem. Soc. Japan*, **7**, 121 (1931).

[8] H. Brückner, "Biochemie des Tabaks," Verlagsbuchhandlung Paul Parey, Berlin, 1936, p. 212.

[9] A. Bomer, A. Juckenack und J. Tillamans, *Handbuch der Lebensmittel-Chemie*, **VI**, 310 (1934).

[10] S. Sabetay and J. Panouse, *Compt. rend.*, **25**, 887 (1947); *C. A.*, 4527 (1948).

[11] W. Halle and E. Pribano, *Ber.*, **47**, 1294 (1914).

[12] S. Sabetay, G. Igolen and L. Palfray, *Rev. Chem. Ind. (Paris)* **50**, 47-81 (1941); *C. A.*, 3413 (1946).

[13] V.N. Shirokaya, *Shorn Rabot Khim Tabak, Bull.*, **125**, 151 (1935); *C. A.*, 4993 (1936).

[14] C. Neuberg und J. Burkard, *Biochem. Z.*, **243**, 472 (1931).

[15] R.L. Shriner and R.C. Fuson, "The Systematic Identification of Organic Compounds," John Wiley & Sons. Inc. New York, 1948, p. 157.

[16] I. Ōnishi and K. Yamamoto, This Bulletin.

Fractionation of Essential Oils into Bases, Acids, Phenols, Carbonyl and Neutral Compounds:

The fractionation scheme is shown diagramatically in Table I.

The yield and properties of each fraction are shown in Table II.

Separation of Acid Fraction by Column Chromatography:

When sodium bicarbonate solution of acid fraction (Table I) was acidified with 80% sulfuric acid, oily substances (Acid I) were separated onto the aqueous solution which might be less soluble components of organic acids. Aqueous layer was extracted with ether (Acid II). Ethereal solution of both fractions was dried over anhydrous sodium sulfate. Removal of ether left Acid I and II fractions which contained considerable amount of dark colored resinous substances. To remove the impurities, Acids I and II were extracted with isooctane. These isooctane soluble fractions were used to separate organic acids by column chromatography. Acid II contained considerable amount of acetic acid and was distilled at atmospheric pressure to collect acetic acid (1,797 mg, first drop b.p. 124°C). The suitable amount of Acids I and II was placed on a column (400 × 18 mm, diam.) of silicic acid[17] using 750 ml. isooctane as the developer according to Vandenheuvel and Hayes's method[18], pressurel 0–20 cm (Hg) was applied from compressed nitrogen gas to force the eluant through the column at a rate of about 2–3 ml. per minute. The effluant is collected at a interval of 5 ml. and titrated with 0.02 N aqueous sodium hydroxide. The chromatograms of Acid I and Acid II are shown in Fig. I and Fig. II, respectively.

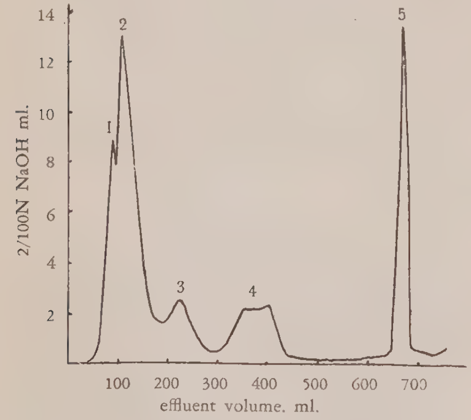

Fig. I. Chromatographic Separation of Acid I. (ca. 200 mg)

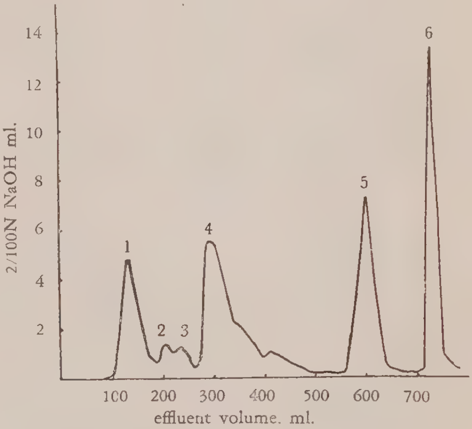

Fig. II. Chromatographic Separation of Acid II. (ca. 400 mg)

The yield of each isolated organic acid is shown in Table III.

Identification of Organic Acids:

Each organic acid isolated by column chromatography was derived to its *p*-bromphenacyl ester[15] and identified by its melting point, microanalysis and infrared spectrum. The spectra of the derivatives were obtained with a Perkin-Elmer Model

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[17] Mallincrodt Analytical Reagent, No. 2847 (100 mesh, specially prepared for chromatography)

[18] F.A. Vandenheuvel and E.R. Hayes, *Anal. Chem.*, **24**, 960 (1952).

Table III

Yield of isolated organic acids from 96 kg of Tobacco Leaves

				Yield (mg) /96 kg leaves	Yield (mg) /kg of leaves	% for total acids
Acid I (10.339 g)	isooctane soluble (6.425 g)	1	*n*-caproic	183	1.9	3.5
		2	unidentified	1014	10.6	19.4
		3	benzoic	331	3.4	6.2
		4	phenylacetic	672	7.0	12.9
		5	formic	undetermined but very few		
	isooctane insoluble (0.288 g)					
Acid II (35.167 g)	isooctane soluble (19.440 g)	1				
		2	isobutyric	81	0.8	1.5
		3				
		4	phenylacetic			
			trans-crotonic	387	4.0	7.3
		5	acetic	2569	26.8	49.2
		6				
	isooctane insoluble (0.834 g)					

21 infrared spectrophotometer installed in our Research Institute.

a) Acid I–1:

p-Bromphenacyl ester (m.p. 63–5°C)[19], *Anal.* Found: C, 54.51; H, 5.15. Calcd. for $C_{14}H_{17}O\ Br$ (*n*-caproic acid derivative): C, 53.71; H. 5.44. The amount of the derivative was so small that we could not recrystallized. Infrared spectrum agreed with *n*-caproic acid *p*-bromphenacyl ester (m.p. 72°C).

b) Acid I–3 and Acid II–3:

p-Bromphenacyl ester (m.p. 118–9°C) showed no depression of melting point when mixed with authentic benzoic acid *p*-bromphenacyl ester (m.p. 119°C), and infrared spectra of both derivatives agreed well with each other.

c) Acid I–4:

p-Bromphenacyl ester (m.p. 89°C) did not depress the melting point of the derivative of authentic phenylacetic acid (m.p. 89°C), and infrared spectra of both derivatives agreed well with each other.

d) Acid II–2:

p-Bromphenacyl ester (m p. 65–8°C), *Anal.* Found: C, 51.44; H, 4.25. Calcd. for $C_{12}H_{13}O_3Br$ (isobutyric acid derivative): C, 50.57; H, 4.56. Infrared spectrum of the derivative agreed with isobutyric acid *p*-bromphenacyl ester (m.p. 77°C).

e) Acid II–5:

p-Bromphenacyl ester (m.p. 85°C) showed no depression of melting point when mixed with the derivative of authentic acetic acid and infrared spectra of both derivatives agreed well with each other.

f) Acid I–5 and Acid II–6:

The majority of the derivative of these fractions was not obtained as crystal but a small part became crystal (m.p. 139–40°C) which did not depress the melting point of the derivative of authentic formic acid (m.p. 142–3°C). Infrared spectrum of the derivative agreed with that of formic acid *p*-bromphenacyl ester.

g) Acid II–4:

Infrared spectrum of the derivative (m.p. 55–70°C) indicate that this derivative was the mixture of *trans*-crotonic acid and phenylacetic acid. Acid

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[19] all melting points are not corrected.

II–4, isolated by chromatographic procedure described previously[17)], was rechromatographed by Marvel and Rands's method[20)] using chloroform-butanol as the developer. This procedure permitted the separation of two organic acids. The first was identified as phenylacetic acid by the method described previously. *p*-Bromphenacyl ester of the second (m.p. 95–6°C) showed no depression of melting point when mixed with the derivative of authentic *trans*-crotonic acid (m.p. 96°C) and infrared spectra of both derivatives agreed well with each other. *Anal*. Found: C, 50.28; H, 3.81. Calcd. for $C_{12}H_{11}O_3Br$ (*trans*-crotonic acid derivative) C, 50.88; H, 3.88.

h) Acid II–1 and Acid I–2:

The derivatives of these fractions were agreed well with each other by comparison of melting point (m.p. 37°C) and infrared spectrum. The melting point of this derivative agreed with *p*-bromphenacyl ester of β-methylvaleric acid isolated by Sabetay and Panouse[10)], but the value of microanalysis showed that of a pentoic acid. *Anal*. Found: C, 52.25; H, 4.88. Calcd. for $C_{13}H_{15}O_3Br$: C, 52.17; H, 5.02. This organic acid is now investigated.

Conclusion

Acetic acid (50%), a pentoic acid (20%) and phenylacetic acid (13%) are the main components in the Acid fraction of steam distillable compounds of Virginia tobacco leaves (Table III). These organic acids have intense odor and might be responsible to the aroma and taste of smoking tobacco. The ester of phenylacetic acid, especially, is one of the perfumes that is useful for tobacco flavour. *trans*-Crotonic acid is the first unsaturated fatty acid isolated from tobacco leaves and much similar to tiglic acid in odor and chemical structure which might to be presented in the condensed water of tobacco toasting machine.

Acknowledgement

The authors wish to express our heartfelt thanks to Prof. Dr. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo for his wise direction and kind guidance and to Director T. Kato, The Central Research Institute, Japan Monopoly Corporation for his strong support to us. We are indebted to Dr. S. Tamura, Department of Agricultural Chemistry, University of Tokyo, for the microanalysis and Mr. T. Miyazawa, Department of Chemistry, University of Tokyo for the infrared analysis and Miss T. Sato and Mr. K. Saito for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. Dr. T. Yabuta for his sound advice.

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20) C.S. Marvel and R.D. Rands, *J. Am. Chem. Soc.*, **72**, 2642 (1950).

Studies on the Essential Oils of Tabacco Leaves

Part II Carbonyl Fraction

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This work was undertaken to separate and identify the carbonyl compounds of the essential oil from Virginia tobacco leaves. In this paper, the carbonyl compounds were separated by the chromatographic adsorption on silicic acid and celite from petroleum benzine-ether and -benzen and identified by melting point and infrared spectra. Acetaldehyde, isobutyraldehyde, benzaldehyde, furfural and 5-methylfurfural were identified from the mixture of 2,4-dinitrophenylhydrazones of carbonyl compounds. Furfural formed two isomers of 2,4-dinitrophenylhydrazones melting at 206°C and 227°C, respectively.

Introduction

The study of carbonyl compounds in the essential oil from tobacco leaves was an almost entirely neglected portion in the tobacco chemistry. Schmuck[1] and Kurilo[2] found only furfural by steam distillation of the tobacco leaves, while from the smokes of the cigarette Neuberg and Burkard[3] found formaldehyde, acetaldehyde, butyraldehyde, benzaldehyde, diethylketone and dipropylketone, and then Neuberg and Kobel[4] found diacetyl and methylethylglyoxal.

In our study, the carbonyl compounds were all derived to 2,4-dinitrophenylhydrazone and separated by the chromatographic adsorption. Since this method was introduced by Strain[5] in 1935, many varieties of adsorbents have been used such as alumina, silicic acid, magnesium sulfate, bentonite, zinc carbonate, filter paper, acetylated paper, filter paper containing silicic acid, silane, calcium sulfate and chromatostrip. In this work, silicic acid and celite, reported by Gorden et al.[6] were used as the adsorbent. For identification of the 2,4-dinitrophenylhydrazones, the use of the infrared spectra proved most effective. The spectra of the 2,4-dinitrophenylhydrazones were obtained with a Perkin Elmer Model 21 recording infrared spectrophotometer. Each derivative was prepared for scanning by grinding the crystals to fine powder and mulling with a nujol. The range was from 1800 cm^{-1} to 650 cm^{-1}. For the determination of the melting points, the micro melting point determination apparatus with microscope was used.

Experimental

Preparation of Derivatives: An ether solution containing the carbonyl compounds of the

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[1] A. Schmuck, "Die Chemie des Tabaks und des Rotabaks," Krasnodar, 1930; H. Brückner, "Biochemie des Tabaks," Verlagsbuchhandlung Paul Parey, Berlin, 1936.

[2] M.E. Kurilo, *State Inst. Tobacco Investigations* (*U.S.S.R.*), No. **90**, 72-80 (1932).

[3] C. Neuberg und J. Burkard, *Biochem. Z.*, **243**, 472 (1931).

[4] C. Neuberg und M. Kobel, *Biochem. Z.*, **275**, 339 (1934).

[5] H.H. Strain, *J. Am. Chem. Soc.*, **57**, 758 (1935).

[6] B.E. Gordon, F. Wopat, Jr., H.D. Burnham and L.G. Jones, Jr., *Anal. Chem.*, **23**, 1754 (1951).

Table I

Chromatographic Separation of Mixture of 2,4-Dinitrophenylhydrazones from Low Boiling Point Carbonyl Compounds in the Essential Oils of Virginia Tobacco Leaves. (Sample I, 160 mg)

Band No.	Yield (mg)	Compounds (2,4-DNPH)	Color
5	4	Unknown	Red
4	17	Furfural (*cis*) Furfural (*trans*)	Red Yellow
3	130	Acetaldehyde	Yellow
2	4 1	5-Methylfurfural Benzaldehyde	Orange Yellow Orange Yellow
1	3	Isobutyraldehyde	Yellow

essential oil of Virginia tobacco leaves, which was separated as described in Part I, was washed twice with water (400 ml.) and dried with sodium sulfate. Two thousand six hundred milliliter of this ether solution was placed into a 1000 ml. two-necked flask attached to washing bottle, in which 1.5 g of 2,4-dinitrophenylhydrazine dissolved in 150 ml. of 95% ethyl alcohol, containing 7 ml. of concentrated sulfuric acid and 10 ml. of water, were put together. By passing nitrogen gas through the said washing bottle, which caught the volatile carbonyl compounds, the ether evaporation was controlled so as to remain a small amount of the solvent under a little reduced pressure at room temperature. The precipitate of the 2,4-dinitrophenylhydrazone containing most part of the low boiling point carbonyl compounds from the washing bottle, yielded 0.72 g (I), having a color of orange-yellow. The liquid left in the two-necked flask was dissolved with 50 ml. of 95% ethyl alcohol and then 1.5 g of 2,4-dinitrophenylhydrazine dissolved in 120 ml. of ethyl alcohol containing 7 ml of concentrated sulfuric acid and 10 ml. of water were added slowly with stirring. Deep red precipitates which were 2,4-dinitrophenylhydrazones, containing most parts of the high boiling point carbonyl compounds, formed almost immediately and yielded 1.23 g (II). (The ethyl alcohol used has been boiled with zinc and potassium hydroxide.)

Chromatographic Separation of (I): Silicic acid[7] and Celite Analytical Filter-Aid[8] mixed at the rates of 2 : 1 in weight was used as adsorbent and dried at 140°C for 24 hrs. in a large evaporating dish, and it was added in 2 to 3 increments to a column (36 × 200 mm) and was firmly tamped with the glass plunger. These additions were repeated until the column was filled to depth of about 20 cm and a filter paper was placed on the top of the adsorbent; then 40 mg (I) dissolved in 20 ml. of chloroform, was poured on the column. After adsorption of the chloroform solution, 50 ml. of petroleum benzine and then 250 ml. of the petroleum benzine containing ether of 2% (by volume) was poured on the column under a pressure of 25–30 cm. (Hg) of nitrogen. As separation of the bands occured, the ether content of the mixed developer was gradually increased to 6% (by volume). Repeating four times this procedure, 160 mg (I) was treated. The results obtained are showed in Table I.

Bands No. 1 and 2 were collected separately in each flask, the effluent corresponding to each colored band by developing. Bands No. 3, 4 and 5 were eluted with ether after the column was extracted and sectioned. The solvent of each fraction was removed by heating in an air stream untill the dry 2,4-dinitrophenylhydrazone remained in the flask.

Identification of Band No. 1: Two successive recrystallization from 60% ethyl alcohol gave yellow crystals. This infrared spectrum agreed completely with that of the authentic isobutyraldehyde 2,4-dinitrophenylhydrazone, and the mixed melting point of this derivative (m.p. 182°C) with authentic derivative (m.p. 186°C) did not depress.

Separation and Identification of Band No. 2: The separation of orange yellow crystals melting

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7) Mallincrodt Analytical Reagent, No. 2847 (100 mesh, specially prepared for chromatography).

8) Johns-Manville.

Table II

Chromatographic Separation of Mixture of 2,4-Dinitrophenylhydrazones from High Boiling Point Carbonyl Compounds in the Essential Oils of Virginia Tobacco Leaves. (Sample II, 90 mg)

Band No.	Yield (mg)	Compounds (2,4-DNPH)	Color
7 6 5 4	14	Unknown	Red
3	30	Furfural (*trans*)	Red
2	2	Unknown	Red
	26	Furfural (*cis*)	Yellow
1	13	5-Methylfurfural	Orange Yellow
	3	Benzaldehyde	Orange Yellow

at 189–90°C were repeated by the same chromatography, after that melting point was raised to 202°C. The infrared spectrum was like that of the authentic 5-methylfurfural 2,4-dinitrophenylhydrazone, but did not agree well. It seemed that this crystals contained a small amount of another derivative. Therefore, the separation of this mixture was attempted by developing with petroleum benzine-benzene (1 : 1 by volume) on the same adsorbent. Two bands could be well separated within the column. The bottom band yielded orange yellow crystals which were recrystallized from ethyl alcohol-ethyl acetate (1 : 2 by volume); the mixed melting point of this derivative (m.p. 241–2°C) with authentic benzaldehyde 2,4-dinitrophenylhydrazone (m.p. 243°C) did not depress, and both infrared spectra agreed completely with each other. While the top band yielded yellow crystals which were recrystallized from ethyl alcohol-ethyl acetate (1 : 1 by volume); the mixed melting point of this derivative (m.p. 220°C) with authentic 5-methylfurfural 2,4-dinitrophenylhydrazone (m.p. 223°C) did not depress, and both infrared spectra agreed completly with each other.

Identification of Band No. 3: Four recrystallization from 60% ethyl alcohol gave yellow crystals, the mixed melting point of this derivative (m.p. 159°C) with authentic acetaldehyde 2,4-dinitrophenylhydrazone (m.p. 164°C) did not depress, and then both infrared spectra agreed with each other.

Separation and Identification of Band No. 4: Band No. 4 containing red and yellow crystals was repeatedly separated by the same chromatography, and three bands were obtained. From the bottom band, acetaldehyde-2,4-dinitrophenylhydrazone was identified by infrared spectrum and melting point. The middle band yielded yellow crystal which was recrystallized from ethyl alcohol-ethyl acetate (1 : 0.5 by volume); the mixed melting point of this derivative (m.p. 206°C) with the authentic furfural 2,4-dinitrophenylhydrazone *cis*-derivative (m.p. 206°C) did not depress, and both infrared spectra agreed completely with each other. The top band yielded red crystals which was recrystallized from ethyl alcohol-ethyl acetate (0.5 : 1 by volume); the mixed melting point of this derivative (m.p. 227°C) with authentic furfural 2,4-dinitrophenylhydrazone *trans*-derivative (m.p. 230–1°C) did not depress, and both infrared spectra agreed well with each other.

Band No. 5: The top band, separated from the column by developing, seems to be a contaminated compound and is not identified yet.

Chromatographic Separation of (II): By previously described procedure, 30 mg (II) were separated. Repeating three times this procedure, 90 mg (II) were treated. The ether content of the mixed developer was gradually increased from 2% to 8% by volume as the separation of the bands occured. The results obtained were shown in Table II.

Band No. I was collected separately in each flask, the effluent corresponding to its colored band by developing. Another bands were eluted with ether after the column was extruded and sectioned. The solvent of each fraction was removed by heating in an air stream until the dry 2,4-dinitrophenylhydrazones remained in the flask.

Identification of Band No. 1: This fraction was the same derivatives with the Band No. 2 of (I) by infrared spectra and melting points, i.e. 5-methylfurfural 2,4-dinitrophenylhydrazone and benzaldehyde 2,4-dinitrophenylhydrazone.

Identification of Band No. 2: This fraction was a mixture of yellow and red crystals, so the separation was repeated by the same chromatography and three bands were obtained. The bottom band agreed with the bottom band of Band No. 4 of (I) by infrared spectrum and melting point, i.e. furfural 2,4-dinitrophenylhydrazone *cis*-derivative. The middle band yielded red crystals, but the amount was not adequate for further research. The top band agreed with the top band of Band No. 4 of (I) and next Band No. 3 by infrared spectrum and melting point, i.e. furfural 2,4-dinitrophenylhydrazone *trans*-derivative.

Identification of Band No. 3: As above described, this fraction agreed with furfural 2,4-dinitrophenylhydrazone *trans*-derivative by infrared spectrum and melting point.

Bands No. 4, 5, 6 and 7: In these fractions, Band No. 7 was not removed by developing. The Bands No. 4, 5 and 6 were obtained by developers employing ether content increased from 8% to 10% by volume. Each amount was not adequate for further research.

Results and Discussion

The separation of 2,4-dinitrophenylhydrazone by chromatographic adsorption on silicic acid and celite gave very good results, but when the amount of sample was in excess, it gave incomplete separation. When the amount of a pure derivative was less than 10 mg, a column of 36×200 mm gave good separation. As described above, when the developing was repeated, and the band was separated again, it showed that the amount of the sample was in excess. It is a flaw of this method that the sample must be of a small amount.

And we must pay the attention to that 2,4-dinitrophenylhydrazones form isomers as furfural 2,4-dinitrophenylhydrazone in this work. We could separate each of two forms of the furfural 2,4-dinitrophenylhydrazone by chromatography on silicic acid and celite. About isomer of the furfural 2,4-dinitrophenylhydrazone, it had previously been described by Braddock et al.[9]. The isomer of 5-methylfurfural 2,4-dinitrophenylhydrazone cannot be separated yet at the present time.

In this work, acetaldehyde 2,4-dinitrophenylhydrazone gave melting point 157°C, but the authentic acetaldehyde 2,4-dinitrophenylhydrazone gave melting point 164°C. This had been explained by Braddock et al.[9] as follows; variations in the temperature of the condensation were found to have little effect upon the melting point of 2,4-dinitrophenylhydrazone of the most compounds, and in that of the acetaldehyde, some great difference was noted. When prepared at 20°C, it melts at 157°C; and when prepared at 55°C, it melts at 166°C. In our work, (I) was prepared at room temperature about 20°C, but authentic acetaldehyde derivative was prepared in water bath. However, both derivatives gave an identical infrared spectrum.

It seems that furfural and 5-methylfurfural did not exist in the tobacco leaves but might be formed from the carbohydrates in the leaves by steam distillation. If so, it has been presumed that the components as hydroxymethylfurfural and levulinic acid might be contained in the essential oils of the tobacco leaves, but we could not separate them at the present time. In order to solve this question, we are now making a plan for extraction of the tobacco leaves with a organic solvent. If the furfural and 5-methylfurfural have been formed from the carbohydrate, those not present in the extract.

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[9] L.I. Bradoock, K.Y. Garlow, L.I. Grim, A.F. Kirkpatrick, S.W. Pease, A.J. Pollaned, E.F. Price, T. L. Reissmann, H.A. Rose and M.L. Willard, *Anal. Chem.*, **25**, 301 (1953).

Now, our attention will be drawn to the presence of other phenolic carbonyl compounds in the phenol fraction described in Part I. On the other hand, concerning the acidic carbonyl compounds, it seems that they have not presented in the acid fraction described in Part I because its reaction with 2,4-dinitrophenylhydrazine was negative.

The contents of these carbonyl compounds were shown in Table III.

Table II

Contents of Carbonyl Compounds found in the Essential Oil of Virginia Tobacco Leaves.

Furfural	50.8%
Acetaldehyde	20.2
5-Methylfurfural	12.8
Benzaldehyde	2.9
Isobutyraldehyde	0.6
Unknown	12.3

The carbonyl compounds are very few in the Essential Oil tobacco leaves, but all these compounds in carbonyl fraction have strong odor, certainly influencing for smoking aroma of tobacco.

Acknowledgement

The authors wish to express our heartfelt thanks to Prof. Dr. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo for his wise direction and kind guidance and to Director T. Kato, the Central Research Institute, Japan Monopoly Corporation, for his strong support to us. We are indebted to Dr. S. Tamura, Department of Agricultural Chemistry, University of Tokyo for the microanalysis and Mr. T. Miyazawa, Department of Chemistry, University of Tokyo for the infrared analysis and Miss K. Yamamoto and Mr. K. Saito for their cooperation in carrying out this experiment. Last but not least, we are also greatly indebted to Emeritus Prof. Dr. T. Yabuta for his sound advice.

Studies on the Essential Oils of Tobacco Leaves

Part III Phenol Fraction

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Received April 28, 1955

An investigation was conducted on the phenolic compounds in the essential oil of Virginia tobacco leaves. Phenol, guaiacol, salicyl aldehyde, methylsalicylate, lauric acid, myristic acid and palmitic acid were isolated and identified. Unknown low boiling substance and some carbonyl compounds were isolated through this work, and were also investigated.

Introduction

Previously, Thoms[1] isolated a sort of phenolic substance from the tobacco leaves, and Sabetay et al.[2,3] isolated eugenol and isoeugenol from the floweres of N. Tabacum. Piatniski[4] reported the presence of eugenol and isoeugenol as the volatile oil in the tobacco leaves, but did not clarify the details. Recently, Rayburn et al.[5] have identified and determined phenol, guaiacol, *m*- and *o*-cresol in the cigarette smoke but they have never reported the presence of phenolic compound in the essential oil of the tobacco leaves. In this paper, we have isolated the phenols by fractional distillation, followed by paper chromatography, preparing azoxy compound of each distillate. The phenol mixture was separated by liquid chromatography using alumina column and eluted by petroleum benzine, ether, ethyl acetate, ethanol and potassium hydroxide successively. The final identification of phenols was accomplished on melting point, infrared spectrum and elementary analysis of their aryloxy-acetic acid and 2,4-dinitrophenylhydrazone, respectively.

Methyl salicylate was identified by the paper chromatography of its hydroxamic acid and the melting point of its *p*-bromphenacyl ester of free acid. Esterification and fractional distillation were undertaken for the isolation of carboxylic acids. Lauric acid, myristic acid and palmitic acid, derived to *p*-bromphenacyl ester, were separated and identified by melting point, infrared spectrum and elementary analysis.

Experimental

Sample: As being described in previous paper[6], 85 kg Virginia tobacco leaves were steam distillated. After saturated with sodium chloride, the distillate was shaken with ether and the ether solution was extracted with aqueous 3% potassium hydroxide solution. The aqueous extract, neutralized with sulfuric acid and saturated with sodium chloride, was shaken with ether repeatedly and thus crude phenols were obtained after removing saturated sodium bicarbonate soluble substances from the ether solution. d^{20} 0.8844; yield 16.351 g (0.019% for leaf) bright red, solidified at room temperature. The crude phenols were put into the fractional distillation under the nitrogen stream. The results are shown in Table I.

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[1] H. Thoms, *Ber. Deutsch. Pharm. Ges.*, **10**, 19 (1900).

[2] S. Sabetay, G. Igolen and L. Palfray, *Compt. rend.*, **213**, 805 (1914).

[3] S. Sabetay, G. Igolen and L. Palfray, *Rev. chim. ind.* (*Paris*) **50**, 47 (1914).

[4] M. Piatniski, *Pub. State Inst. Tobacco and Makhorka Ind., Krasnodar, U.S.S.R. Bull.*, **49** and **51** (1929); **81** (1931).

[5] C.H. Rayburn, W.R. Harlan and H.R. Hanmer, *Anal. Chem.*, **25**, 1419 (1953).

[6] I. Ōnishi and K. Yamasaki, This Bulletin.

Detection of Phenols by Paper Chromatography: The presence of phenols on infrared spectra of fraction 4 being found, further research on the phenolic compound in each fraction was undertaken by paper chromatography, preparing their oxyazocompounds according to Hossfeld et al.[7), 8)]. Five milligrams of each fraction was dissolved in 0.6 ml. of aqueous 8% sodium hydroxide. A few drops of diazotized sulfanilic acid were added in this solution. After acidified with an excess of hydrochloric acid, the reaction mixture was extracted once with benzene. The aqueous solution was diluted with alcohol and spotted on the position at 5 cm from the below end of the filter paper, saturated with aqueous 4% sodium carbonate, and dried.

Table I

Fractional Distillation of Phenol Fraction in the Essential Oil of Virginia Tobacco Leaves

Fraction	b.p. (°C)	Bath T. (°C)	Yield (g)
1	–36	– 86	0.9758
2	–70	112–134	0.2732
3	–80	140–157	0.1603
4 4.2 mm. (Hg)	67–70	170–175	0.0700
5 ,,	78–80	190–200	0.0259
6 ,,	–90	–250	0.3234
Residue			11.3201

Table II

Paper Chromatography of Phenol-oxyazo-compounds.

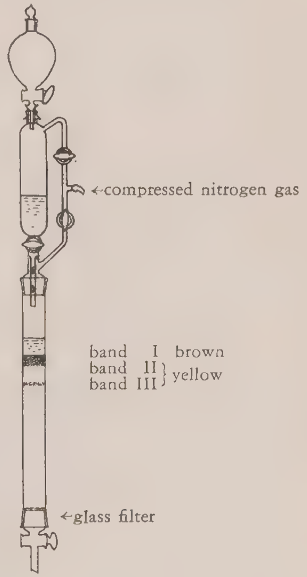
Fraction	*Rf* value and color
1	—
2	reddish orange colored
3	reddish orange colored
4	0.08 (orange), 0.03 (yellow), 0.21 (yellow)
5	0.09 (yellow)
6	—
Residue	—

Water saturated *n*-butanol (water saturated *sec*-butanol for fraction 5) was used for irrigating solvent and developed for about 16 hrs. at room temperature. The results are shown in Table II.

Fraction 1 was considered mostly to be the ether which remained at previous distillation. Fractions 2 and 3 were very volatile, colorless and transparant liquid having a violent odor. Though they showed slightly positive color test with ferric chloride and diazo reagent, no phenolic compound having such a low boiling point is known. On standing still for many days after they were mixed with phenylisocyanate in a closed vessel, no precipitate was formed.

As three clear spots were detected from the fraction 4, liquid chromatography was followed by.

The Isolation and Identification of the Phenolic Compounds from Fraction 4 by Liquid Chromatography: Fraction 4 was dissolved in 20 ml. ether, added 10 ml. petroleum benzine (b.p. 40–65°C) and poured into the 2×20 cm alumina chromato tube[9)]. Three bands shown in Fig. I appeared.



Adsorbent: Al_2O_3

Fig. I Column Chromatogram of F. 4

[7)] R.L. Hossfeld, *J. Am. Chem. Soc.*, **73**, 852 (1951).

[8)] Wen-Hua-Chang and R.L. Hossfeld, *J. Am. Chem. Soc.*, **74**, 5766 (1952).

[9)] T. Yokozuka, *J. Agr. Chem. Soc. Japan*, **27**, 276 (1953).

Table III

Liquid Chromatographic Separation of Phenol

Fraction	*Rf* and Color of oxyazo compound	Color test			Yield (g)
		$FeCl_3$	Diazo	Fuchsin	
Petr. benzine	—	—	-	-	—
Ether	- -	−	−	+	0.049
Ethyl acetate	0.13 (yellow) 0.08 (orange)	+	+	−	0.038
Ethanol	0.08 (orange)	+	+	−	0.036
NaOH	0.21 (yellow)	+	+	+	0.021

Then they were eluted, employing petroleum benzine (b.p. 40–65°C), ether, ethyl acetate, ethanol and aqueous 0.5% sodium hydroxide successively. A pressure was applied from compressed ntirogen gas to make the eluting velocity constant. Diazo reagent was added to a small portion of each 5 ml. effluent and the color positive fractions were collected as follows:

a) Eluted with 150 ml. of petroleum benzine. The effluent was colorless, reaction negative and band III descended a little.

b) On pouring ether, band III descended rapidly and colored orange. The effluent first bore bright yellow, then turned to colorless, and reaction was negative. It needed 200 ml. of solvent.

c) On eluting with 150 ml. of ethyl acetate, both I and II bands were not descended and the effluent was colorless, and reaction was positive.

d) Eluted with 150 ml. of ethanol. Band II spread and the color faded. The effluent colored yellowish and reaction was positive.

e) On eluting with alkali hydroxide, both I and II bands were descended together and the effluent colored fresh yellow. Three hundred milliliter of alkali solution was used just before band I eluted out.

Solvent was distilled off from each effluent under reduced pressure and nitrogen stream (the alkali solution was acidified, extracted with ether, washed with water, dried and evaporated the solvent). The characterization of each fraction by paper chromatography is shown in Table III.

Ether fraction was a viscous orange yellow oil and had sweet odor like apricot, and formed dark red precipitate on adding calculated 2,4-dinitrophenylhydrazine. By heating, the resultant changed to black powder, uneasy soluble in hot alcohol and easily soluble in ethyl acetate. It consists of at least two carbonyl compounds and further research is now going on.

Identification of Phenol: Thirty five milligrams of yellow oil was obtained from the ethyl acetate fraction, removing the small amount of substance corresponding to *Rf* 0.08 by repeated liquid chromatagraphy using alumina column. Three tenth millimeter of aqueous 33% sodium hydroxide, 0.25 ml. of 50% monochloroacetic acid and 1.5 ml. of water were added to the oil successively. The mixed solution was heated for one hour in a gently boiling waterbath, cooled, diluted with water, acidified to congo-red with sulfuric acid, and extracted with 3% sodium carbonate. On acidification of this alkali extract and standing still for a long time in cool gave a precipitate, which was filtered off and recrystallized from hot water. Twelve and three tenth milliliter colorless plate crystal yielded. The mixed melting point of this derivative (m.p. 95.5°C) with phenoxy acetic acid (m.p. 96°C) did not depress. Infrared spectra agreed decidedly with each other. *Anal.* Found: C, 63, 40 ; H, 6.00. Calcd. for $C_8H_8O_3$: C, 63.15 ; H, 5.26.

Identification of Guaiacol: Thirty two milligrams of orange oil from the ethanol fraction was induced to the derivative as described above. Six and a half milligrams white needle crystal yielded. The crystallization of this derivative was very hard and it was inclined to decompose to a brown oil by recrystallization. This melting point (116.5°C) depresses 3–4°C by an admixture of the authentic of guaiacol (m.p. 120°C), but infrared spectrum and analytical figures were identical together. *Anal.* Found: C, 59.33 : H, 5.41. Calcd. for $C_9H_{10}O_4$: C, 59.34 ; H, 5.49

Identification of Salicyl Aldehyde : As the yellow oil obtained from the sodium hydroxide fraction evidently showed the color reaction of aldehyde, it was induced to 2,4-dinitrophenylhydrazone and recrystallized from alcohol. It gave 21.5 mg of red, fine needle crystal, decomposed at 252°C. The mixed melting point with the authentic of salicyl aldehyde did not depress and infrared spectra was identical with each other. *Anal.* Found : C, 51.33 ; H, 3.67 ; N, 18.24. Calcd. for $C_{14}H_{10}O_3N_4$: C, 51.66 ; H, 3.31 ; N, 18.54.

Identification of Methylsalicylate : Fraction 5 of crude phenol was a brown oil, having violent salol odor and found the presence of ester on the infrared spectra. The warm petroleum benzine (b.p. 40–60°C) soluble portion was decolorized, dried and yellow oil remained in distilling off the solvent. One spot (*Rf* 0.91) was detected in paper chromatography of its hydroxamic acid[10], which was identical with the authentic of methylsalicylate. After saponified of fraction 5 a yellowish white precipitate, subliming at 138–145°C, was obtained as an acid. The melting point of its *p*-bromphenacyl ester, a white fine needle, was 140°C and agreed with the authentic of salicylic acid.

Identification of Lauric acid : Fraction 6 of crude phenol was a yellow oil and coagulated to a brown mass on standing still in the air. A yellowish white solid substance was obtained from the aqueous 3% sodium carbonate soluble portion of fraction 6. It had slightly acidic odor and showed the presence of carboxylic compound on its infrared spectra. On fractional precipitation of its *p*-bromphenacyl ester, two crystals were obtained, each melting point and infrared spectra obserbed. The former is probably lauric acid according to the melting point (80.6°C) and infrared spectra, though analytical figure showed a some difference between carbon percentage with theoretical. *Anal.* Found : C, 61.67 ; H, 7.31. Calcd. for $C_{20}H_{29}O_3Br$: C, 60.45 ; H, 7.30. The latter may be a mixture of two derivatives.

Esterification of the Distillation Residue : The distillation residue of crude phenol did not show the color test of phenol, but the presence of high molecular fatty acid on the infrared spectra. Reddish, solid reaction product (12.93 g) was obtained by esterification with methanol and sulfuric acid as usual.

The result of fractional distillation of methylester is shown in Table IV.

Table IV
Fractional Distillation of Methylester from Distillation Residue of Phenol

Fraction	b.p. (°C) /2.5 mm. (Hg)	Bath T. (°C)	Yield (g)
1	– 60	–173	0.025
2	128–130	200–217	0.078
3	155 constant	226–256	8.834
Residue			2.65

Fraction 1 was a yellow oil, not indicated apparent boiling point. Acidic substance (12.5 mg) was obtained after saponified, but no precipitate of its *p*-bromphenacyl ester.

Identification of Myristic Acid : Fraction 2 coagulated partially at room temperature. After saponified the ester and acidified the resulting alkali solution, 32 mg (m.p. 54°C) white mass separated and filtered off, followed by washing with water, recrystallized from alcohol and dried. The melting point of its *p*-bromphenacyl ester was 81.5°C and did not depress by mixing with the authentic of myristic acid (m.p. 82°C). Infrared spectra was agreed with, too. *Anal.* Found : C, 62.05 ; H, 7.90. Calcd. for $C_{23}H_{33}O_3Br$: C, 62.05 ; H, 7.76.

Identification of Palmitic Acid : Fraction 3 was the main portion of ester and gave white crystal at room temperature. The melting point of methyl ester was 29°C, free acid 62°C and that of *p*-bromphenacyl ester 85.5°C. According to these points, it is certain that this is palmitic acid.

Liquid Chromatography of the Ester Distillation Residue : The distillation residue of ester was dissolved in warm benzene and poured into the 2×20 cm calcium phosphate chromato tube and eluted with benzene, followed by chloroform. The eluted benzene solution colored yellow and palmitic acid was obtained by the procedure as described above (0.962 g). Dark brown resinous substance (0.924 g) was obtained on distilling off the chloroform. The precipitate, separated on cooling the hot alcohol soluble portion, was filtered off and to this filtrate a sulfuric alcohol solution of 2,4-dinitrophenylhydrazine was added. Orange yellow precipitate was formed immediately, filtered off and dried (39 mg). On these carbonyl compounds, further investigation is now going on.

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[10] Y. Inoue and M. Noda, *J. Agr. Chem. Soc. Japan*, **24**, 291 (1950)

Acknowledgment

The authors wish to express our heartfelt thanks to Prof. Dr. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo for his wise direction and kind guidance and to Director T. Katō, The Central Research Institute, Japan Monopoly Corporation for his strong support to us. We are indebted to Dr. S. Tamura, Department of Agricultural Chemistry, University of Tokyo for the microanalysis and Mr. T. Miyazawa, Department of Chemistry, University of Tokyo for the infrared analysis and Miss R. Shimizu and Mr. K. Saitō for their cooperation in carrying out this experiment. Last but not least, we are also indebted to Emeritus Prof. Dr. T. Yabuta for his sound advice.

Enzymatic Resolution of Racemic Amino Acids

Part III. Purification of Mold Acylase and Resolution of DL-Phenylalanine and DL-Methionine

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In this paper further purification procedures for the preparation of mold acylase and the enzymatic susceptibility of acylated amino acids are described, together with the resolution of DL-phenylalanine and DL-methionine.

The mold acylase of *P. vinaceous* was prepared by fractionation with 0.6 saturation of ammonium sulfate, acetone precipitation, calcium gel adsorption and following elution with phosphate buffer. The enzyme was more effective toward the acylderivatives of aromatic substituted amino acids than toward those of the aliphatic amino acids and it had little or no action upon acylated D-amino acids. By utilizing the optical specificity of the enzyme, DL-phenylalanine and DL-methionine were resolved to the optical enantiomorphs.

Introduction

The hog kidney acylase has been studied to a considerable extent in the laboratory[1, 2, 3, 4] of the National Cancer Institute, U.S.A. and Neuberg and Mandl[5] noted that the acylderivatives of DL-amino acids are asymmetrically hydrolyzed by commercial amidase preparations, Pancreatin and *Aspergillus oryzae* enzyme (Takadiastase).

It has been previously reporetd by the present authors[6, 7] that a number of strains of *Aspergillus* and *Penicillium* produce an enzyme to hydrolyze acylated racemic amino acids asymmetrically and the partially purified acylase can be employed for the resolution of acylated racemic amino acids.

The present investigation is an extension of the previous studies. The more active acylase was prepared by the further purification procedure with adsorption with calcium gel and elution with phosphate buffer and it enabled to extend the resolution of DL-phenylalanine and DL-methionine, and the assay of susceptibility of N-acylated amino acids and glycylpeptides towards the mold acylase.

Experiment

Preliminary Experiment (Fractional Precipitation with Ammonium Sulfate)—Two g of the crude enzyme powder which was prepared by fractionation with saturation of ammonium sulfate and acetone precipitation as described in the previous paper[7] was dissolved in 40 ml. of distilled water and salted out with the equal

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[1] S.M. Birnbaum, L. Levintow, R.B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

[2] K. R. Rao, S. M. Birnbaum, R.B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

[3] S.C.J. Fu, and S.M. Birnbaum, *J. Am. Chem. Soc.*, **75**, 918 (1953).

[4] W.S. Fones and M. Lee, *J. Biol. Chem.*, **201**,847(1953).

[5] C. Neuberg and I. Mandl, *Enzymologia*, **14**, 128(1950).

[6] K. Michi and H. Nonaka, *J. Agr. Chem. Soc. Japan*, **28**, 343 (1954).

[7] K. Michi and H. Nonaka, *J. Agr. Chem. Soc. Japan*, **28**, 346 (1954).

Table I. Rates of Hydrolysis with Acylase I and Acylase II

Substrate	Rate* of Hydrolysis with Acylase I		Acylase II
	0.25 mg	0.5 mg	1.5 mg
	O. D.	O. D.	O. D.
Acetyl-DL-phenylalanine	.420		.430
Acetyl-DL-glutamic acid		.465	.107
Glycyl-L-leucine		.180	.045

* Rate was expressed by the optical density of ninhydrin measurement of the liberated amino acid with 0.25 mg and 0.5 mg of Acylase I, and 1.5 mg of Acylase II respectively.

volume of saturated ammonium sulfate solution at pH 6.5. The centrifuged precipitates (Acylase I) were treated with acetone as described in the previous paper7). The mother liquor from separation of Acylase I was saturated with ammonium sulfate at pH 6.5. The resulting precipitates (Acylase II) were treated with acetone.

Hydrolytic activity of Acylase I and Acylase II fractions were measured by the photometric ninhydrin method described later in determination of initial hydrolytic rates of the acylated amino acids with exception of 40 mM substrate and two hours incubation at 37°. Table I. shows the result.

Preparation of Enzyme—One kg. of the molded wheat bran which was inoculated with *P. vinaceous* and incubated for five days at 25° as described in the previous paper7) was homogenized with 5000 ml. of water in a mixer and allowed to stand for one hour with occasional stirring. The homogenate was strained through cotton cloth and filtered on Kieselguhr by suction. The filtrate was adjusted to pH 6.5 by addition of dilute ammonia. The resulting sediment was discarded by filtration on Kieselguhr. The filtrate was concentrated to 2000 ml. under reduced pressure in a water bath of 45–50°.

The concentrated extract was treated with 450 g. of solid ammonium sulfate per 1000 ml. of the extract in a cold bath of 0°C. under careful adjustment of pH 6.0–7.0 by neutralization with dilute ammonia during addition of the salt. The resulting precipitates which contain most of the activity were separated by centrifugation.

The precipitates were dissolved in 100 ml. of distilled water and dialyzed against cold distilled water in a refrigerator overnight. The contents of the dialysis sack were centrifuged to remove precipitates which separated during the dialysis. The solution was treated with 1.2 volumes of chilled acetone in a cold bath at 0°∼−10°C. and the mixture was allowed to stand in a refrigerator overnight.

The centrifuged precipitates were dissolved in 1000 ml. of cold distilled water and the enzyme was adsorbed on calcium phosphate gel8) which prepared from 1000 ml. of 0.66 M calcium chloride and 1000 ml. of 0.44 M dibasic sodium phosphate, by shaking for one hour at pH 6–6.5. The adsorption was followed by centrifugation of the adsorption complex. The supernatent liquid was now discarded and the enzyme was eluted from the adsorbate by stirring with 500 ml. of phosphate buffer of pH 7–7.2 for thirty minutes. After centrifuging the insoluble material, the eluate was poured into a beaker and salted out with 300 g. of ammonium sulfate under careful adjustment of pH and temperature as described above.

The centrifuged precipitates were dissolved in 50 ml. of cold distilled water and dialyzed overnight in a refrigerator. The fractional precipitation with 1.2 volumes of chilled acetone was employed again. The acetone precipitates were stored under the acetone solution in a refrigerator. The precipitates were centrifuged and dried in vacuo over phosphorus pentoxide or it might be dissolved in water and quickly lyophilized under high vacuum. The weight of the dried preparation was about 0.5 g.

Substrates for Enzymatic Assays—The glycylderivatives of glycine, L-leucine, and the L and D isomers of phenylalanine were prepared by condensation9),10) of the phthalyl-glycylchloride

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8) E.G. Ball, Biochemical Preparations, Vol. 2, p. 18(1952).

9) K. Yamashita and Y. Sahashi, *J. Agr. Chem. Soc. Japan*, **28**, 672 (1954).

10) K. Yamashita and M. Yashiro, *J. Agr. Chem. Soc. Japan*, **28**, 674 (1954).

and the amino acid in glacial acetic acid with the presence of sodium acetate, and liberation of phthalyl group by refluxing with hydrazin hydrate.

These include glycylglycine, m.p. 217°; glycyl-L-leucine, m.p. 247°, $[\alpha]_D^{20}=-35.12°$, N-calculated 14.89, found 14.92%; glycyl-L-phenylalanine, m.p. 260°, $[\alpha]_D^{20}=+41.5°$, N-calculated 12.62, found 12.55%; glycyl-D-phenylalanine, m.p. 257°, $[\alpha]_D^{20}=-41.6°$, N-calculated 12.62, found 12.62%, and L-leucinamide, m.p. 98°, $[\alpha]_D^{14}=+8.4°$ (2% in H_2O), N-calculated 21.5, found 21.21%.

All of the rotation value cited except leucinmide were obtained on 1.6 per cent aqueous solution.

Substrates for Resolution

(1) Acetyl-DL-phenylalanine: Acetyl-DL-phenylalanine was synthesized by catalytic hydrogenation of acetaminocinnamic acid which prepared from acetylglycine and benzaldehyde, m.p. 146°.

(2) Acetyl-DL-methionine: DL-Methionine was acetylated in the usual manner with acetic anhydride in glacial acetic acid, yielding product which after crystallization from water, possessed m.p. 112°.

Enzymatic Assay—Figure 1. shows the effect of pH for the hydrolysis of acetylphenylalanine and acetylmethionine. The pH optima for the hydrolysis of these acylated amino acids with the acylase are about 7.8 and 7.4 respectively.

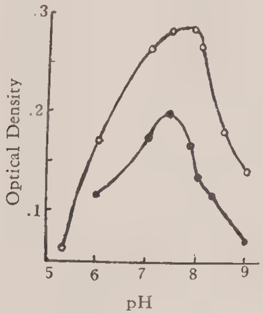

Fig. 1. Effect of pH on the Mold Acylase

○—○ : Acetyl-DL-phenylalanine
●—● : Acetyl-DL-methionine

Initial rates of hydrolysis of N-acylated amino acids, glycylpeptides and L-leucinamide with the mold acylase were determined as follows. Digests were composed of 0.5 ml. of acylase solution in water, 1 ml. of 0.1 M phosphate buffer and 0.5 ml. of 0.5 M substrate for acetylphenylalanine, acetylmethionine and chloroacetylvaline; 0.08 M substrate for acetylglutamic acid and acetylalanine, and 0.02 M substrate for peptides. After incubation of each digest at 38° for one hour the digest was diluted to suitable volume (2 mM solution of susceptible substrate).

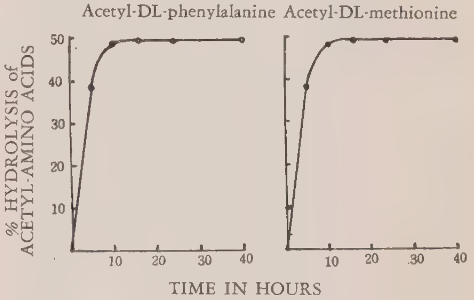
Acetyl-DL-phenylalanine Acetyl-DL-methionine

Fig. 2. The asymmetric hydrolysis of N-acetyl derivatives of DL-phenylalanine and DL-methionine with mold acylase.

The digests consisted of 0.5 ml. of 20 mM substrate. 1 ml. of buffer, and 0.5 ml. of enzyme solution. Hydrolysis was followed by photometric ninhydrin measurements, as described in the case of determination of initial hydrolytic rates of acylated amino acid.

Samples, 0.2 ml. aliquots, were heated with 1 ml. of the ninhydrin reagent of Stein and Moore[11] for twenty minutes in a vigorously boiling water bath. After dilution of the content with 5 ml. of 50% ethanol, optical density was read by electrophotometer with 10 mm cuvet and 570 mμ filter. The blank was read against the same digest except the inactivated enzyme instead of active acylase.

The amounts of liberated amino acids corresponding to the readings were calculated from each standard curve plotted for 0.5—4 mM amino acid.

The rates of hydrolysis are expressed in terms of micromoles of substrate hydrolyzed per hour per mg. of N of the acylase.

Resolution of Acetyl-DL-phenylalanine (Isolation of L-phenylalanine)—Thirty g. of acetyl-DL-phenylalanine was dissolved in 1000 ml. of water

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[11] S. Moore and W.H. Stein, *J. Biol. Chem.*, **176**, 367 (1948)

Table 2. Initial Hydrolytic Rates* of N-acylated Amino Acids and Glycylpeptides with Acylase

Substrates	Rate of Hydrolysis	Substrates	Rate of Hydrolysis
Acetyl-DL-phenylalanine	3053	Glycyl-L-phenylalanine	126
Acetyl-DL-methionine	1988	Glycyl-L-leucine	70
Chloroacetyl-DL-valine	1012	Glycylglycine	7
Acetyl-DL-alanine	719	Glycyl-D-phenylalanine	7
Acetyl-DL-glutamic acid	333	D-Leucinamide	14
Acetyl-D-phenylalanine	0		
Acetyl-D-glutamic acid	0		

* The digests were incubated at 38° for one hour at each optimum pH for acylated amino acids and at pH 7 for peptides and an amide.

and neutralized by addition of 10 g. of calcium-carbonate to pH 7. To the aqueous solution 70 mg. of acylase was added to insure complete hydrolysis of the L-form of the acetylderivative and the mixture was incubated at 38° under toluene for two days.

At the end of the digestion period, the calculated amounts of oxalic acid in water was added to the digest to remove calcium. The filtrate of the calcium oxalate on charcoal was shaken with 2 g. of norit for one hour to remove enzyme protein. The filtrate was evaporated under reduced pressure to a very low bulk. The residue was warmed with 300 ml. of alcohol and allowed to stand in a refrigerator overnight. The L-amino acid precipitated was filtered by suction and washed with alcohol. The L-phenylalanine weighed 10 g. (83% of theory). The compound recrystallized from water possesses m.p. 276°, and $[\alpha]_D^{20}=-34.8°$ *Analysis.* Found: N, 8.49. Calcd for $C_9H_{11}O_2N$, N: 8.48%.

Isolation of D-Phenylalanine—The mother liquor of the L-amino acid was evaporated to dryness and the residue taken up in absolute alcohol was allowed to stand in a refrigerator overnight. After filtration of the small amount of resulting precipitates the filtrate was evaporated in vacuo and the residue dissolved in a little water was placed in a refrigerator overnight. Acetyl-D-phenylalanine crystallized out of the solution. The yield of the ninhydrin-negative material melting at 168° was 11.5 g.

After refluxing acetyl-D-phenylalanine with 70 ml. of 2 N HCl for two hours the mixture was evaporated in vacuo. The residue was dissolved in the minimum amount of water and neutralized with 3 N NaOH to pH 6. The solution was concentrated until crystallization was observed, and placed in a refrigerator. The D-amino acid filtered weighed 8 g. (70% of th.). The purified amino acid by recrystallization from water possesses m.p. 270° and $[\alpha]_D^{20}=+34.6°$ (1.6% in water) *Analysis.* Found: N, 8.54, Calcd. for $C_9H_{11}O_2N$, N: 8.48%.

Resolution of Acetyl-DL-methionine (Isolation of L-Methionine)—Twenty g. of acetyl-DL-methionine was dissolved in 800 ml. of water and added 6 g. of calcium carbonate to neutralize to pH 7. The acylase (70 mg.) in water was added to the solution and the mixture was incubated under toluene at 38° for two days.

At the end of the digestion, the mixture was treated as described in the case of phenylalanine. L-Methionine yielded 6.2 g. (86% of th.). m.p. 275°, $[\alpha]_D^{14}=+23.42°$ (2% in 6 N HCl)

Analysis. Found: N, 9.27, Calcd. for $C_5H_{11}O_2NS$: N, 9.39%.

Isolation of D-Methionine—The mother liquor from the separation of the L-methionine which was almost negative for the ninhydrin reaction was evaporated and the residue was refluxed with 80 ml. of 2 N HCl for two hours. The mixture was evaporated and treated with 3 N NaOH to pH 5.8 as described in the case of phenylalanine. Yield of D-methionine was 4.5 g. (61% of th.), m.p. 270°, $[\alpha]_D^{14}=-23.3°$ (2% in 6 N HCl).

Analysis. Found: N, 9.15, Calcd. for $C_5H_{11}O_2NS$: N, 9.3%.

Discussion

In an assay (Table I) it was noted that the enzyme was salted out by half-saturation with ammonium sulfate. The purification procedure of the mold acylase presented in this paper has been demonstrated that the enzymatic activity is relatively stable to adsorption with calcium gel and elution with phosphate buffer of pH 7. The enzyme was more readily adsorbed from dialyzed rather than nondialyzed enzyme solution and it was stable in the pH range employed in the fractionation procedure. There was a gradual loss of activity at values less than pH 5.5 or greater than pH 7.5. Because of the relatively marked instability of the enzyme towards acetone precipitation and drying, further purification was restricted to recycle these procedures.

Greenstein and his coworkers noted that the N-acylderivatives of the same amino acids as tested here were hydrolyzed with the hog kidney acylase at progressively decreasing rates in the following order:

Acetylmethionine > Acetylglutamic acid
> Chloroacetylvaline > Acetylalanine
> Acetylphenylalanine.

The present authors noted that these acylderivatives were hydrolyzed with the mold acylase at progressively decreasing rates in the following order:

Acetylphenylalanine > Acetylmethionine
> Chloroacetylvaline > Acetylalanine
> Acetylglutamic acid.

The glycylpeptides were attacked with the acylase at progressively decreasing rates in the following order:

Glycyl-L-phenylalanine
> Glycyl-L-leucine > Glycylglycine.

In general the mold acylase was more effective towards the acylderivatives of aromatic-substituted amino acids than towards those of aliphatic amino acids.

The mold acylase has little capacity to hydrolyze glycyldipeptides which contain the D-amino acid in the chain and has almost no capacity to hydrolyze acetyl-D-amino acids. As the results seen in Fig. 2 show a maximal hydrolysis of exactly 50 per cent, it is apparent that the mold acylase hydrolyzes only the acylderivatives of L-amino acid and has almost no action on those of D-amino acid. Since the acylase possesses a marked optical specificity, it can be employed for resolution of racemic amino acids. The enzyme hydrolyzes only the L isomer of acylated DL-amino acid and the liberated L-isomer is readily separated from acylated D-amino acid by virtue of the alcohol solubility of the latter and the alcohol insolubility of the free amino acid.

It may be suggested that the mold acylase is a carboxypeptidase, for it requires the presence of a free α-carboxyl group in the susceptible substrate.

Acknowledgments

The authors are grateful to Mr. Kinoshita of the Ajinomoto Company for assistance in the preparation of the enzyme, to Miss Isobe for performing some of the microanalyses and to Dr. S. Abe of the Microbiology Institute of Tokyo University for kindly supplying *P. vinaceous.* The research wasa ided in part by a grant from the Ajinomoto Company.

Summary

1. The mold acylase of *P. vinaceous* was prepared by fractionation procedures with 0.6 saturation of ammonium sulfate, acetone precipitation, calcium gel adsorp-

tion and following elution with phosphate buffer.

2. The enzymatic susceptibility of N-acylated amino acids and glycylpeptides towards the acylase were studied. The enzyme was more effective towards the acylderivatives of aromatic-substituted amino acids than towards those of aliphatic amino acids. The enzyme possessed little or no action to hydrolyze the acylated D-amino acids.

3. DL-Phenylalanine and DL-methionine were resolved in relatively good yield by asymmetric hydrolysis of the acetylderivatives of these amino acids with the mold acylase.

NOTE

Studies on Chrysanthemic Acids. Part II.

A Separation of (±)-trans-Chrysanthemic Acid from the Mixture of (±)-trans and (±)-cis Chrysanthemic Acids.

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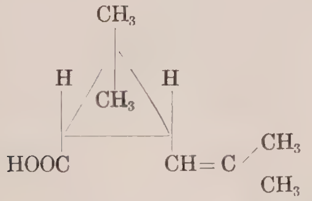
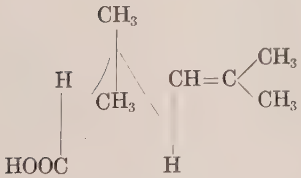
Faculty of Agriculture, University of Tokyo

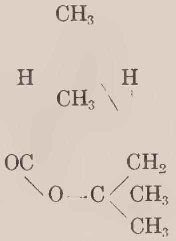
Received April 30, 1955

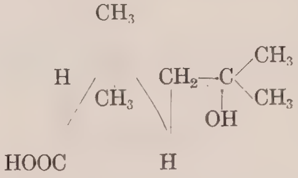
The addition of 2,5-dimethyl-2,4-hexadiene to ethyl diazoacetate easily affords a mixture of ethyl esters of (±)-*trans* and (±)-*cis*-chrysanthemic acids. A mixture of free (±)-*trans* and (±)-*cis*-chrysanthemic acids is obtained by hydrolysis of the ester with alkali[1]. Usually the higher melting *cis*-acid (racemic form of I), crystallizes at first and the *trans* acid (II) crystallizes gradually. The former is less soluble in ethyl acetate and can be separated in pure state by recrystallization with considerable ease. Whereas the preparation of pure *trans*-acid by means of recrystallization is not so successful owing to contamination of the *cis*-acid. The present authors offer a convenient method for preparing the pure *trans*-acid which is based upon the experiments of S.H. Harper[2].


(I)


(II)

The starting crude *trans*-acid is roughly separated from the *cis*-isomer by filtration after warming the mixed crystalline mass of the *cis*- and the *trans*-acids at about 60°C to melt the latter. The crude *trans*-acid is boiled with diluted sulfuric acid and is turned to (±)-*trans*-α-hydroxydihydrochrysanthemic acid whicy is water-soluble but light-petroleum-insoluble and (±)-dihydrochrysanthemolactone (III) which is formed from the *cis* acid. The pure hydroxy acid (IV) is obtained by ether extration and recrystallization. The


(III)


(IV)

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[1] I. G. M. Campbell and S. H. Harper J. Chem. Soc., **1945** 2833 S. H. Harper, H. W. B. Reed and R. A. Thompson, *J. Sci., Food Agric.*, **1951** 14.

[2] S. H. Harper and R. A. Thompson, *J. Sci., Food Agric.*, **1952** 230.

pure hydroxy acid affords the pure *trans*-acid by distillation in the presence of catalytic amount of *p*-toluenesulfonic acid followed by redistillation under reduced pressure.

The authors express their thanks to professor Y. Sumiki for his interrest in this work.

Experimental

Crude (±)-*trans*-chrysanthemic acid (90 g) contaminated with (±)-*cis*-acid is refluxed with 6 l of 5% (weight) sulfuric acid under stirring for 3 hrs. The cooled mixture is extracted with light petroleum (40–60°C). (Dihydrochrysanthemolactone[2] and impure *trans*-acid are obtained from the light petroleum extract.) The aqueous layer is continuously extracted with ether by percolation during 36 hrs. Evaporation of the ether extract gives (±)-*trans*-δ-hydroxy dihydrochrysanthemic acid (56 g) as needles, m.p. 94–95°C. Recrystallization from ethyl acetate-light petroleum gives the hydroxy acid (43 g) m.p. 96°C.

The hydroxy acid (40 g) is distilled in the presence of ca. 20 mg of *p*-toluenesulfonic acid under reduced pressure. The pure (±)-*trans*-chrysanthemic acid (35 g) is obtained by redistillation (b.p. 142°C/12 mm) which soon solidifies in the receiver and melts at 54°C.

COMMUNICATION

On a New Water-Soluble Ergot Alkaloid, Molliclavine

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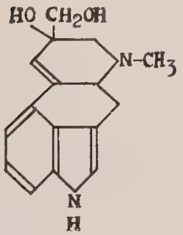
The Laboratory of the Institute for Fermentation attached to Takeda Pharmaceutical Industries, Ltd. Osaka, Japan

Received July 4, 1955

Previously, Abe et al.[1] isolated penniclavine (formula I) and a new alkaloid triseclavine (formula II) from the saprophytic culture of the ergot fungus parasitic on *Elymus mollis* Trin. growing in Japan.

Recently, we have found that the elymoclavine fraction separated by the starch-column partition chromatography using a mixture of *n*-butanol-acetic acid-water as its solvent from the total alkaloids of the sclerotia or saprophytic culture of the fungus above-mentioned contained an unknown alkaloid of the penniclavine- or triseclavine-type. We have succeeded in isolating this alkaloid from the saprophytic culture of the said fungus in very small yield. The new alkaloid, for which we propose the name molliclavine, was crystallized from benzene, chloroform, ethyl acetate, acetone or methanol in prisms; m.p. 253° (uncorr. decomp.), $[\alpha]_D^{17}=+30°$, $[\alpha]_{5461}^{17}=+42°$ (c. 0.2 in pyridine). *Anal.* Found: C, 70.92; H, 6.86; N, 10.08. Calcd. for $C_{16}H_{18}O_2N_2$: C, 71.09; H, 6.71; N, 10.36%. It gave a green color with van Urk's and Keller's reagents, but a deep yellow color with concd. sulfuric acid. The ultraviolet absorption spectrum of molliclavine showed maxima at the vicinity of 226, 287 and 294mμ., and almost agreed with those of agroclavine and elymoclavine. It was insoluble in petroleum ether; very sparingly soluble in benzene, toluene, chloroform and carbon tetrachloride, sparingly soluble in ethyl acetate and butyl acetate; moderately soluble in acetone, but readily soluble in methanol, ethanol and pyridine. It exhibited almost on fluorescence in any organic solvent. It dissolved appreciably in water, and readily in dilute acids. From its solutions in various acids it was precipitated by phosphotungstic acid, Mayer's reagent and other alkaloidal reagents. On catalytic hydrogenation, it yielded the dihydroderivative, which gave colors undistinguishable from those given by agroclavine, elymoclavine or lysergic acid with van Urk's reagent, Keller's reagent and concd. sulfuric acid.



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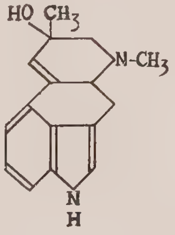

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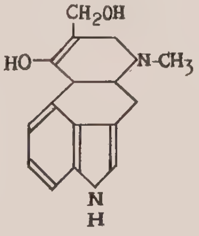

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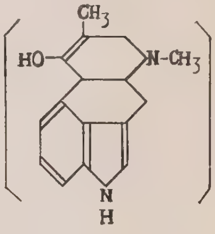

IV

From these data, it was provisionally concluded that molliclavine might be represented by formula III. Formerly, we[2] were able to demonstrate by chemical experiments that penniclavine and triseclavine were produced through elymoclavine and agroclavine, respectively. We are confident that, considering the quantitative relation between elymoclavine and molliclavine present in the sclerotia or saprophytic cultures of various ergot fungi, molliclavine is also produced through elymoclavine.

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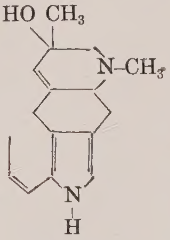
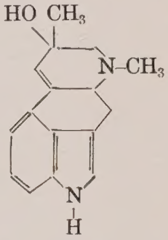
[1] M. Abe, S. Yamatodani, T. Yamano and M. Kusumoto, *Bull. Agr. Chem. Soc. Japan* (communication), **19**, 92 (1955).

[2] S. Yamatodani and M. Abe, *ibid.* (communication), **19**, 94 (1955).

Under these circumstances, it is probable that the sclerotia as well as the saprophytic culture of a certain ergot fungus actually contain an alkaloid, probably representable by formula IV.

Part of this work was reported at the Annual Meeting of Agr. Chem. Soc. Japan held at University of Tokyo on March 31, 1955.

Additions and Corrections
Volume 19, No 1, 1955

page	column	line	for	read
15	Synopsis	1	glucouse	glucose
18	left	4	sa memedium	same medium
21	left	22	, is would	, it would
69	Synopsis	8	17.8%	19.3%
〃	〃	9	41.9%	42.9%
73	Table III	(Pentosan)	0.0603 g	0.0652 g
〃	〃	〃	17.8%	19.3%
〃	left	7	0.018 g	0.0018 g
〃	right	4–5	(0.6870 − 0.5101)/0.3874 × 100/89.9 = 70.6%.	0.2459/0.3874 × 100/89.9 = 70.6%, where 0.2459 is the weight (g) of galactose equivalent to (0.687° − 0.5101) g of mucic acid.
76	Table VII,	3 2	52.2[1]	53.1[1]
〃	〃	3 4	34.9	35.8
〃	〃	4 4	46.5 4)	47.6[4]
〃	〃	4 5	41.5 5)	42.9[5]
〃	〃	Note 1	52.2	53.1
〃	〃	Note 4	34.9 × 1.33 = 46.5	35.8 × 1.33 = 47.6
〃	〃	Note 5	46.5 × 0.90 = 41.9	47.6 × 0.90 = 42.9
〃	left	13	41.9%	42.9%
〃	〃	21	17.8%	19.3%
〃	〃	23	41.9%	42.9%
93	formula II			
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